A NEW PHOSPHOGALACTOLIPID

MEMBRANE COMPONENT OF BIFIDOBACTERIUM BIFIDUM VAR. PENNSYLVANICUS

F. W. VAN SCHAIK

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PROMOTOR: Dr. J. H. VEERKAMP
CO - PROMOTOR: PROF. DR. S. L. BONTING

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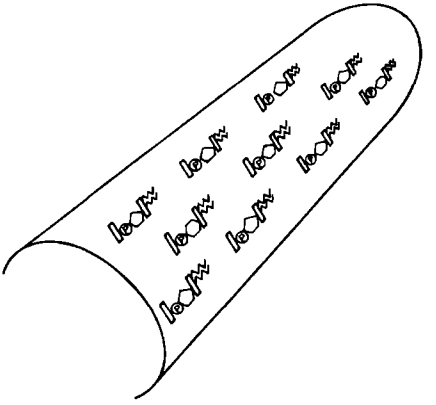
PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. MR. F. J. F. M. DUYNSTEE VOLGENS HET BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 26 SEPTEMBER 1974 DES NAMIDDAGS TE 4 UUR

DOOR
FRANS WILLEM VAN SCHAIK
GEBOREN TE DRIEBERGEN

DRUK: KRIPS REPRO B.V. MEPPEL

Indien de meest eenvoudige vorm van
leven zó geschapen is, met hoeveel
zorg heeft God dan de mens gemaakt



Aan mijn ouders
Aan Heleen, Marion en Harriëtte

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ABBREVIATIONS

A 550	absorbance at 550 nanometer
ADP	adenosine triphosphate
by vol.	by volume
cpm	counts per minute
CDP	cytidine diphosphate
CL	cardiolipin
DNA	deoxyribonucleic acid
et al.	et altera (and other authors)
GPG	glycerophosphorylglycerol
GPGPG	di-glycerophosphoryl glycerol
GPGalG	glycerophosphorylgalactosyl glycerol
k	fractional turnover rate
Lipid-15	glycerophosphorylgalactosyl diglyceride
Lyso-15	glycerophosphorylgalactosyl monoglyceride
Lyso-CL	lyso-cardiolipin (mon-, di-, triacyl-GPGPG)
mCi	millicurie
MPi	mobility toward inorganic phosphate during electrophoresis
NAD^+	oxidized nicotinamide-adenine dinucleotide
NADH	reduced nicotinamide-adenine dinucleotide
^{32}P	radioactive phosphorus isotope
PA	phosphatidic acid
Pi	inorganic phosphate
PG	phosphatidyl glycerol
Rf	mobility relative to front during chromatography
RNA	ribonucleic acid
ox,red	oxidation followed by reduction
^{35}S	radioactive sulphur isotope
sn	stereospecific numbering
Tt	turnover time
U	units

The purpose of this thesis is to extend the knowledge of bacterial membranes, particularly that of *Bifidobacterium bifidum* var.*pennsylvanicus*. The vital importance of the cell membrane for life in all biological systems is now generally being recognized. The cytoplasmic membrane provides a protective barrier between the external environment and the internal milieu of the cell. Moreover, membranes function as catalytic surfaces, providing a hydrophobic environment, where the components of multienzyme complexes may interact with a high degree of efficiency.

To achieve a satisfactory understanding of the functioning of the cell membrane its detailed molecular composition and structure must be known. Phospholipids are essential components of all membranes.

This thesis is concerned with the phosphate-containing lipids of *Bifidobacterium bifidum* var.*pennsylvanicus*. Originally, this organism was introduced in our laboratory for the investigation of its cell wall synthesis, which is inhibited by the absence of certain glucosamine derivatives. The shape of the cell is a curved rod, but in unfavourable culture media it is often branched to give the characteristic Y-shape which led to the designation of "bifid". Investigations of the membrane have been prompted by the idea that the morphological changes could be effected by structural changes in the membrane occurring as a result of cell wall inhibition.

The current knowledge of function, composition and assembly of bacterial membranes have been summarized in Chapter 1. Chapter 2 deals with the general experimental procedures in our study of the membrane lipids of *B.bifidum* var.*pennsylvanicus*. Characterization of the phosphate-containing lipids of this organism has revealed the presence of hitherto unknown phosphogalactolipids, the molecular structure of which has been elucidated (chapter 3). Changes in the phospho(galacto)lipid composition of the organism in response to different environmental conditions have been investigated (chapter 4). The last chapter (5) deals with the rates of renewal (turnover) of the individual phosphate-containing lipids and with possible precursor-product relationship between these lipids.

THE BACTERIAL MEMBRANE

1.1. History of *Bifidobacterium bifidum* var. *pennsylvanicus*.

The study of the bacterial membrane in this thesis is confined to the phosphate-containing lipids of *B. bifidum* var. *pennsylvanicus*. A brief summary of earlier investigations of this organism and of other *Bifidobacterium* species is therefore in order.

The different morphological shapes of the *Bifidobacterium* species, i.e. curved rods and branced cells, were first observed by Tissier in 1899 in stools of breast-fed babies. Low Ca^{2++} concentration in the culture medium can induce bifid formation, while addition of calciumchloride to such cultures is accompanied by a reversion to the bacilloid form (Kojima et al, 1973). An appropriate selective medium for the primary isolation of these organisms became available only in 1950. The biology of *Bifidobacteria* has recently been summarized (Poupard et al, 1973).

Bifidobacterium bifidum var. *pennsylvanicus* has been isolated in 1953 from stools of infants and the vaginal secretions of pregnant women. This organism requires human milk for growth. The essential factor in human milk (bifidus factor) has been identified as N-acetyl-D-glucosamine-containing saccharides, which are lacking in bovine milk (Gyorgy and Rose, 1955). These growth factors are shown to be utilized by this organism as a substrate for cell wall synthesis (O'Brien et al, 1960). Certain N-substituted derivatives of D-glucosamine also promote growth of the organism (Lambert and Zilliken, 1965).

Before 1963 the designation *Lactobacillus bifidus* was in common use, but since then *Bifidobacterium* has been recognized as a separate genus of the family *Lactobacillaceae* by the discovery of the phosphoketolase pathway for the fermentation of hexoses (Scardovi and Trovatelli, 1965, De Vries and Stouthamer, 1967, Veerkamp, 1969). In this new fermentative pathway both fructose-6-phosphate phosphoketolase and xylulose-5-phosphate-phosphoketolase play important roles Aldolase and glucose-6-phosphate dehydrogenase could not be detected in extracts. This rules out the presence of the glycolytic system and the hexose monophosphate shunt, which are characteristic pathways of the genus *Lactobacillus*. Other taxonomic differences between both genera occur in the DNA-, phospholipid-, and fatty acid compositions. The guanine-cytosine contents of twenty-eight *Bifidobacterium* strains range from 57 to 69 per cent, while these contents are below 50 per cent in *Lactobacillus* (Sebald et al, 1965). A comparative study of the phosphate-containing lipids have demonstrated that cardiolipin and phosphatidylglycerol are common phospholipids in both genera.

All *Bifidobacterium* strains investigated contain an unknown phospholipid, designated as compound-15 (Exterkate et al, 1971a). This lipid was thought to be glycerophosphorylglyceroldiacylphosphatidylglycerol, but this structure proved to be untenable when reinvestigation revealed galactose as a structural part of this lipid (Veerkamp and Van Schaik, 1974). The differences in relative amounts of the various acids, particularly lactobacillic acid, are also significant. Lactobacillic acid accounts for less than 5% of the fatty acids in *Bifidobacterium* strains and for more than 15% in *Lactobacillus* strains in the stationary phase (Veerkamp, 1971).

1.2. Cellular architecture of bacteria.

Bacteria have a rigid cell wall varying in thickness from 0.15 to 0.8 μ. Investigations during the past decades have revealed that the heteropolymers peptidoglycans account for the rigidity of the cell wall. (Osborn, 1969; Reaveley and Burge, 1972). These substances have the structure of an organized network and account for as much as 25-90% of the dry weight of the cell wall of Gram-positive and 5-20% of that of Gram-negative bacteria. The designations Gram-positive and -negative refer to the staining reaction developed by Gram in 1884. This reaction distinguishes between two chemically different kinds of cell walls. Gram-negative bacteria have a multi-layered structure as revealed by electron microscopy. The innermost layer of the wall consists of peptidoglycan, also called murein or glycopeptide. Overlying this is a wider layer made up of loosely packed protein molecules, which is itself covered by a layer of lipopolysaccharide. The cell walls of Gram-positive bacteria are made up of peptidoglycans, polysaccharides and often teichoic acids. Their unilayer structure suggests that these materials are very intimately associated in this case. Many bacteria are surrounded by capsules of polysaccharides that are external to the rigid cell wall (Fig. 1).

The cytoplasmic membrane, which is localized inside the rigid cell wall, forms the osmotic barrier between the cytoplasm and the external environment. Treatment of rod-shaped Gram-positive bacteria with lysozyme degrades the cell wall and transforms the cells in many cases into osmotically fragile spheres, termed protoplasts. Invaginations of the cytoplasmic membrane occur and are called mesosomes. The wide-spread occurrence of the mesosomes in both Gram-positive and Gram-negative bacteria suggests that it is a common feature of bacterial ultrastructure (Reusch and Burger, 1973). The difference with the intracytoplasmic membranes in higher organisms is, that the bacterial mesosome maintains contact with the extracytoplasmic space through the cell wall (Fig. 1). On account of the absence of a membrane surrounding the nucleus, bacteria belong to the procaryotic organisms.

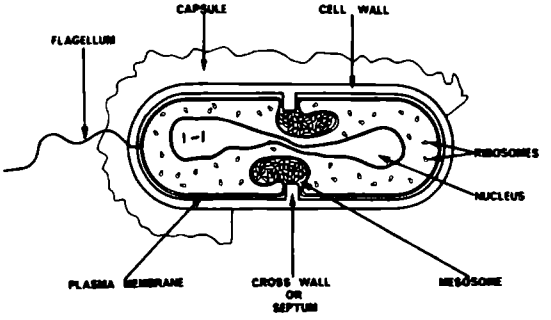

Figure 1.

Cellular architecture of a Gram-positive bacterium (from Salton, 1971).

1.3. Membrane composition.

In thin sections, the cytoplasmic membrane of the Gram-positive bacterium normally presents the characteristic trilamellar appearance typical of all biological membranes (Korn, 1969; Reaveley and Burge, 1972). Danielli and Davson proposed a membrane model in 1935, in which a lipid bilayer is covered with protein. The transparent layer observed by electronmicroscopy would represent the lipid bilayer, which is sandwiched between the darker staining layers of protein. The proteins interact ionically with the polar headpieces of the phospholipid molecules in this model, while apolar amino acid side chains interact with the hydrocarbon chains of the phospholipids.

Modifications of this membrane model have been proposed, since this model fails to account for many recent findings. VanderKooi and Green (1970) have proposed that a membrane consists of globular proteins packed in two layers with only few points of contact between adjacent units. In the "fluid mosaic structure"-model, postulated by Singer and Nicolson (1972), the membrane is represented as a two-dimensionally oriented viscous solution. The globular proteins are thought to be partially embedded in and partially protruding from the membrane (Fig. 2). The protruding part has on its surface the ionic residues (- and +) of the protein, while the embedded part contains primarily the nonpolar residues. This dynamic model is consistent with all experimental data available and with thermodynamic restrictions.

The chemical composition of bacterial membranes has been summarized by Op den Kamp et al. (1969) and by Reaveley and Burge (1972). Proteins and lipids account for 40-70% and 10-30% respectively of the dry weight of the membranes of Gram-positive organisms. Other minor constituents are RNA, DNA, polysaccharide. The presence of RNA as well as of polyhydroxybuturate may be due to the contamination of the isolated membranes with ribosomes and polyhydroxybutyrate granules from the cytoplasm. Whereas many details about the bacterial lipids have been elucidated, the study of the membrane proteins and the interaction between lipids and proteins has been started only quite recently.

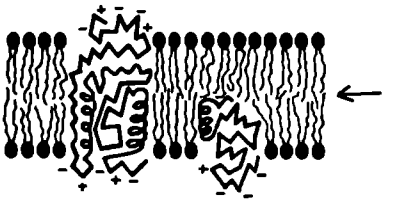

Figure 2

Cross-section of a membrane The phospholipids are arranged as a discontinuous bilayer with their polar heads pointing to the aqueous phase The membrane proteins are shown as globular molecules partially embedded in, and partially protruding from the membrane (from Singer and Nicolson, 1972)

1.4. Bacterial lipids.

Most information about the membrane lipids of bacteria has been derived from studies of the lipid composition of whole cells. In many cases virtually all the cell lipid is present in the cell membrane. The lipid composition differs considerably, not only in procaryotic and eucaryotic cells, but also in different groups of procaryotic organisms (Op den Kamp et al, 1969; Goldfine, 1972). Current knowledge of phospholipids is summarized by Wakil (1970) and by Ansell et al. (1973).

As in higher organisms the lipids with a *sn*-glycerol 3-phosphate*) backbone are found as the most abundant form of polar lipids in bacteria too.

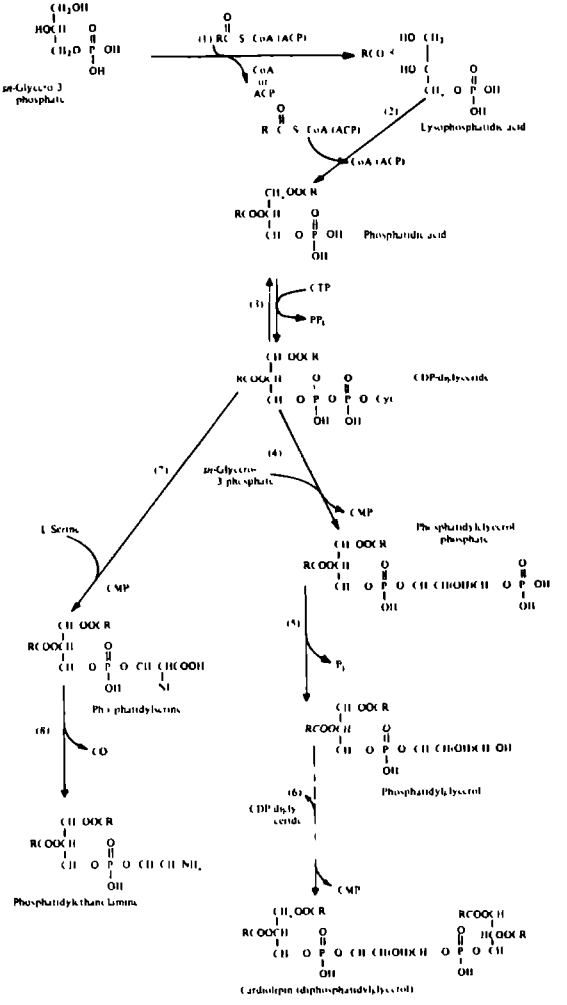

Figure 3.

The biosynthesis of the phospholipids of Escherichia coli (from Bell et al., 1971). ACP stands for acyl carrier protein.

The principal bacterial phospholipids are phosphatidylglycerol, cardiolipin (also termed diphosphatidylglycerol) and phosphatidylethanolamine. The structure of these lipids and their metabolic interrelationship in *Escherichia coli* are given in Fig. 3. Phosphatidylcholine does not generally occur in bacteria, in contrast to plants and animals, which contain high amounts of this phospholipid. Its biosynthesis through a stepwise methylation of phosphatidylethanolamine is the only pathway known so far in bacteria. Lecithin, as well as the "incomplete lecithins" formed after one and two methylation steps, are found in a number of Gram-negative bacteria. Phosphatidylethanolamine is frequently the major phospholipid in Gram-negative organisms, while phosphatidylglycerol and cardiolipin are often predominant in Gram-positive bacteria. Phosphatidylglycerol can function as a precursor for cardiolipin, but it can also be the end product of biosynthesis, just as phosphatidylethanolamine. Typical intermediate lipids are phosphatidylserine, phosphatidylglycerolphosphate, phosphatidic acid and CDP-diglyceride. These lipids do not accumulate in the cell owing to their fast rate of metabolism. CDP-diglyceride is the most important intermediate in the synthesis of both zwitter ionic (nitrogenous) and acidic phospholipids (Fig. 3). The biosynthesis of phosphatidic acid can occur via three different pathways starting from monoglyceride, diglyceride and *sn*-glycero-3-phosphate. The former two compounds are phosphorylated with the help of ATP. The acylation of *sn*-glycero-3-phosphate is shown in Figure 3. The fatty acids introduced in phosphatidic acid are not necessarily conserved in the next steps of biosynthesis. An acylation-reacylation mechanism may enable the bacterium to renew its fatty acids. This mechanism can explain why fatty acid compositions of various phospholipids are deviating from that of the common precursor moledule (de Kruyff et al, 1970).

In halophilic bacteria alkylphospholipids are found, in which fatty alcohols instead of fatty acids are linked to glycerol. Remarkable is the *sn*-glycerol-1-phosphate configuration in the di-O-alkyl analogues of phosphatidylglycerophosphate from *Halobacterium cutirubrum* (Joo and Kates, 1969).

In membranes of Gram-positive bacteria the phospholipids make up 40-70% of the total lipids, while neutral and glycolipids together vary from 25-45% (Reaveley and Burge, 1972). Bacteria generally contain no sterols, which are common in cells of higher organisms. However, some *Mycoplasma* strains, which lack a rigid cell wall, have a requirement for sterols (Razin, 1973). These organisms are parasitic and have ready access to sterols, but are not capable of synthesizing these substances. Free fatty acids, polyisoprenoids, glycerides and poly-β-hydroxy butyric acid are mentioned as neutral lipids in bacteria. The amount of neutral fats per cell is usually low, and mono- and diglycerides are reported more often than triglycerides. Bacteria, do not appear to store glycerides as energy reserves like higher organisms. Poly-β-hydroxybutyric acid, however, appears to serve this function in some bacteria. These polymeric lipids are not membrane bound, but are found as granules in the cells.

*) The prefix "*sn*" stands for stereospecific numbering, according to the nomenclature for glycerol compounds recommended by IUPAC-IUB (Biochim. Biophys. Acta, 152 (1968) 1-9). The configuration, previously designated as D-1 or L-3 has been replaced by *sn*-3 in this nomenclature.

Glycolipids are common membrane constituents, in particular in Gram-positive bacteria (Shaw, 1970). The glycosyldiglycerides are structurally analogous to the phosphoglycerides, because they are composed of carbohydrate residues glycosidically bound to the 3-position of a 1,2 diacyl *sn*-glycerol. The first step of their biosynthesis involves the transfer of a sugar from its appropriate nucleotide derivative to a diglyceride. The carbohydrate moiety of these lipids consists of glucose, mannose or galactose monomers or combinations of these compounds. The biosynthesis of di- and trihexosyldiglycerides occurs through a sequential transfer of the sugars. Mono-, di-, and trigalactosyldiglycerides have been characterized in *B.bifidum* var.*pennsylvanicus* (Exterkate and Veerkamp, 1971; Veerkamp, 1972). UDP-galactose is involved in their biosynthesis (Veerkamp, 1974). In plants galactolipids are found as major lipids of the photosynthetic apparatus.

Phosphoglycolipids are glycolipids with a phosphate containing substituent, while sugar containing phospholipids are preferentially termed glycophospholipids. These lipids are now being isolated from a variety of bacteria (Shaw and Stead, 1972). Prior to 1968 the reports of glycophospholipids were restricted to the family of phosphatidylinositol mannosides and glucosaminylphosphatidylglycerols. In these instances carbohydrate residues are bound glycosidically to phosphatidylinositol and phosphatidylglycerol, respectively. Both terms phosphoglycolipids and glycophospholipids can be used with equal right for compounds such as phosphatidylglucosyldiglyceride, which lipid was isolated from *Pseudomonas diminuta* (Wilkinson and Bell, 1971). Phosphogalactolipids are also found in *B.bifidum* var.*pennsylvanicus*. Their structure has been elucidated by us (Chapter 3).

1.5. Teichoic acids.

Teichoic acids will be treated here on account of the structural similarity of the sugar containing teichoic acids and the water-soluble deacylation product of the phosphogalactolipids from *B.bifidum* var.*pennsylvanicus*. Teichoic acids are water-soluble polymers present in both cell wall and membrane of many Gram-positive bacteria. Membrane teichoic acids are believed to be present in all Gram-positive species (Baddiley, 1972). The backbone of these polymers may be glycerolphosphate, ribitolphosphate or glycerophosphorylhexose (Fig. 4). Membrane teichoic acid from *Lactobacillus fermenti*, has been described (Wicken and Knox, 1970). It has been concluded from the nature of the products of alkaline hydrolysis that the teichoic acid is combined with glycolipids and phospholipids in the membrane.

1.6. Functions of the cytoplasmic membrane.

The importance of the plasma membrane for cell metabolism is generally being recognized (Op den Kamp et al, 1969, Rose, 1970). The absence of intracellular organelles in bacteria dictates that the functions of the membranes of such organelles must be carried out by the cytoplasmic membrane-mesosome system. The following main functions can be discerned: (i). It acts as the osmotic barrier of the cell. (ii). Transport systems are located in the membrane. (iii). The membrane is the site for the biosynthesis of various cellular constituents.

The osmotic barrier can be broken by treatment of the bacterium with lipid solvents, which causes the release of low molecular weight compounds from the organism. This demonstrates that the osmotic barrier resides in the lipid-protein organization of the membrane. Similar findings are obtained

from protoplasts, which are easily plasmolyzed. Still the cytoplasmic membrane does not constitute the only barrier to the passage of solutes from the environment into the micro-organism, for solutes must also pass across the cell wall. The cell wall functions as a heteroporous molecular sieve and also as a weak ion-exchange resin. Often polymers with a molecular weight below about 50.000 are able to penetrate cell walls of Gram-positive bacteria. The binding of cations, especially divalent cations, in the cell wall appears to be a function of teichoic acids (Cutinelli and Galdiero, 1967). Factors other than molecular weight must be important when uptake of DNA molecules takes place in certain bacteria during genetic transformation. The cell walls are then permeable to DNA with an average molecular weight of several millions (Rose, 1970, p.108).

The cytoplasmic membrane acts as an organelle, which concentrates nutrients within the cell and excretes waste products. The types of transport processes can be distinguished as passive diffusion, facilitated diffusion, and active transport (Rose, 1970). Passive diffusion occurs when the driving force is the difference in the concentration (with non-electrolytes), or electrical potential (with ions), across the membrane. Water passes across the membrane by passive diffusion, but this process seems to be of little or no importance for the uptake or excretion of other compounds. The entrance and efflux of most solutes are mediated by specific carrier or transport mechanisms located in the membrane, known as permeases. Facilitated diffusion means a "downhill" transport of solutes catalyzed by a permease. Excretion

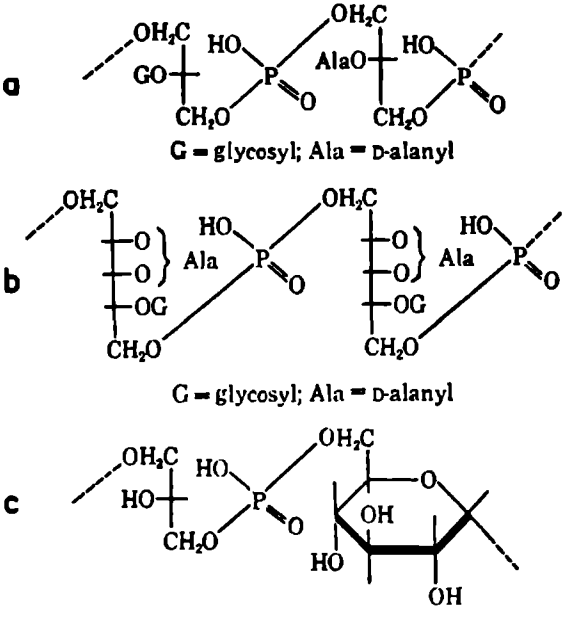

Figure 4.

Different kinds of teichoic acids. a: glycerol teichoic acids, b: ribitol teichoic acid, c: glycerol teichoic acid in which glycosyl residues constitute a part of the polymer. Possible glycosyl and alanyl substituents are also indicated in a and b.

of solutes usually involves downhill transport, which is carrier mediated. Transport against a concentration gradient requires metabolic energy, and is described as active transport. The uptake of most solutes follows this process. Most studies on solute transport through membranes are done with mammalion cells. Probably the best understood transport process in bacteria is the galactoside transport in *E.coli*. There may be as many as 8.000 β-galactoside permease sites on the surface of this organism (Fox et al, 1967), so that the total permease complement of a micro-organism may account for an appreciable fraction of membrane protein.

Recent developments have revealed the role of the bacterial membrane in cell wall formation (Salton, 1971; Lennarz and Scher, 1972). Peptidoglycan and lipopolysaccharide biosynthesis are taking place partly in the cell interior, whereas the final formation of the polymer is an external process. The membrane fulfills an important role in the transport of the intermediates and the attachment of these compounds into the polymers. The intermediates are covalently linked with a C55-isoprenoid alcohol via a pyrophosphate bridge and thus transported through the membrane. These isoprenoid alcohols are also involved in the biosynthesis of several teichoic acids, of polymannan in *Micrococcus lysodeikticus* (Scher and Lennarz, 1969), and of capsular polysaccharide in *Aerobacter aerogenes* (Troy et al, 1971).

The enzymes involved in lipid synthesis also belong to the enzymic machinery of the membrane. The enzymes of phospholipid catabolism in *E.coli* have been found to be localized in the cell wall (Bell et al, 1971). Possibly, the separate localization of anabolic and catabolic enzymes will prove to be valid for other bacterial lipids too. The function of the lipids as co-factor required for enzymic reactions has been summarized by Coleman (1973).

Synthesis of RNA, DNA and protein synthesis occurs at the surface of the bacterial membrane, as is the case for many other intracellular enzymic reactions. It is not clear, whether RNA and DNA present in isolated membrane fractions are real constituents of the bacterial membrane. A possible role of the plasma membrane in replication of bacterial DNA has been proposed by Jacob et al. (1963). In their replicon model they postulated that the binding points of two DNA molecules are moving in opposite directions from the cell equator. In this way an equal distribution of chromosomes into each daughter cell is warranted.

1.7. Assembly of the membrane.

The process of deposition of the various membrane components so as to form the final, highly organized membrane structure is largely unknown. Current knowledge of membrane assembly has been summarized by Rothfield and Finkelstein (1968) and Machtiger and Fox (1973).

Membranes are thought to be repeating arrays of individual lipoprotein subunits. Membrane synthesis by insertion of such subunits has been suggested by reconstitution experiments in which lipid and protein components of the membrane were allowed to aggregate in vitro. A reconstitution of some enzyme systems in functional form has been reported, e.g. the hexose phosphotransferase system in *E.coli* and the glycosyltransferase system that participates in the synthesis of lipopolysaccharide.

If pre-fabricated lipoprotein subunits are inserted in the membrane, one should expect a stringent coordinated synthesis of the constituents. Indeed, a defect in assembly of a functional lactose transport system in *E.coli* has been observed following starvation for an essential fatty acid (Fox, 1969). However, changes in lipid-protein ratio may be obtained without loss of

vital physiological functions of the membrane. E.g., when cells of *M. laidlawii* were treated with chloramphenicol, membrane protein synthesis was inhibited but membrane lipid synthesis was not affected (Kahane and Razin, 1969). Upon removal of the inhibitor the cells started to multiply again. Similarly, cessation of phospholipid biosynthesis in glycerol auxotrophs by starvation of glycerol produces no selective effect on the synthesis of membrane proteins or their association with the membrane These experiments demonstrate that the synthesis of membrane lipids and proteins can be uncoupled to some extent.

Another line of investigation of membrane assembly concerns its topology, i.e. the geographical distribution of constituents over the cell surface (Kepes and Autissier, 1972, Machtiger and Fox, 1973). Newly synthesized material may be inserted into the membrane either in a limited number of growing zones or in growth points distributed all over the membrane surface. A similarity in the manner of membrane and cell wall replication is suggested by their juxtaposition in the cell. Cell wall replication has been investigated using fluorescent-antibody technique (Cole, 1965). In *Streptococci pyogenes* cell wall material is laid down centripetally, so that the future new hemispheres of the new cocci are initiated back-to-back. A similar pattern of membrane replication has been found in *E. coli* (Fig. 5) using a penicillin lysis procedure to discriminate between random versus nonrandom segregation of a functional membrane component (Kepes and Autissier, 1972). An inducible permease was used as a membrane marker. The segregation of parental membrane and non-parental membrane among the descendant is shown in Fig. 5. Two populations of cells could be discerned after three divisions, with and without parental membrane marker. Cells with zones of parental membrane conservation containing the induced permease were lysed more rapidly with penicillin.

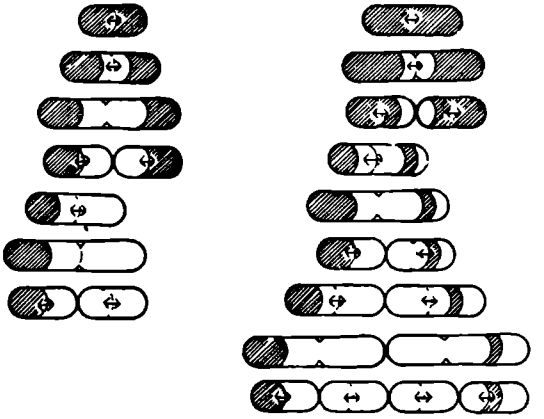

Figure 5

Zones of membrane conservation Distribution of parental membrane among descendants in bacteria with a single median growing zone Permease as parental marker is located in the hatched area Arrows indicate the elongation of new membrane in the growing zone Left, a cell deinduced just after the last cell division Right, deinduction of a cell midway between two cell divisions (from Kepes and Autissier, 1972)

^{32}P-radioactivity was incorporated into the membrane phospholipids during permease induction. Thereafter, the cells were lysed and the decrease of ^{32}P-phospholipids corresponded to the decrease of the cell population containing permease activity. This finding indicates that new phospholipids are deposited in the growing zone in order to separate the zones of parental phospholipids.

However, the presence of one equatorial zone of membrane growth is far from common. Many other studies of membrane topology based on the segregation of membrane proteins and lipids in bacteria indicated that, if zonal membrane growth does occur, the zones are less than 500 Å in linear dimension along the membrane surface. This resembles the manner of cell wall replication in *Salmonella* which approaches a process of diffuse intercalation (Cole, 1965). So far the experimental resolution is too small to establish zonal growth when four or more regions of membrane conservation are present in the cell.

1.8. Membrane synthesis in relation to the cell cycle.

The membrane is presumed to play a role in chromosome segregation and cell division. It is therefore to be expected that membrane synthesis is related in a characteristic fashion to the cell duplication cycle. In order to study membrane synthesis in relation to cell age it is possible to induce bacteria to divide more or less synchronously, i.e. within a very short period of time compared to the generation time. For instance, synchronous growth can be initiated after starvation of an essential nutrient or after termination of DNA synthesis at a non-permissive temperature (Cameron and Padilla, 1966).

The rate of lipid biosynthesis increases near the time of septum formation in *B. megaterium KM* (Daniels, 1969). In *E. coli*, however, no increase in the rate of synthesis of any phospholipid has been observed at any stage of the cell cycle (Ohki, 1972). Although the net synthesis of phosphatidylglycerol increases continuously, the process of renewal (turnover) of this lipid occurs in a step-wise manner, approximately midway through the cell cycle. The appearance of cytochrome b_1 and *sn*-glycerol 3-phosphate transport is also discontinuous in this organism, while the protein content increases steadily during the cell cycle.

In *B. subtilis* a rapid synthesis of membrane protein was found during the latter part of the growth cycle, while no net synthesis takes place during the early part (Sargent, 1973). Phospholipid synthesis is continuous throughout the cell cycle, while the activity of succinic dehydrogenase, a tightly bound marker enzyme of the membrane, increases discontinuously. Apparently, some membrane constituents may be synthesized during the entire cell cycle, whereas others appear at a specific moment of the cell cycle. The step-wise synthesis of succinic dehydrogenase in *B. subtilis* occurs just before cell separation and, therefore, is added in the course of a linear membrane growth period. Sargent postulated that during the period of net synthesis proteins from the cytoplasm are inserted into the membrane, while an efflux of proteins from the membrane occurs during the period of zero net synthesis. The cellular processes dependent upon the cell cycle could be of some importance in membrane assembly, but no causal relationship has been established so far.

EXPERIMENTAL PROCEDURES

The procedures used for the cultivation of *Bifidobacterium bifidum* var. *pennsylvanicus* and for the study of its membrane lipids are described in this chapter. The chemical and enzymic degradation procedures used for the structural investigation of the phosphogalactolipids are described in the next chapter (section 3.2).

2.1. Cultivation of the organism.

The organism, initially isolated by Dr. P. György (Philadelphia, USA) in 1953, was cultivated in the medium according to Norris (Poupard et al; 1973). The medium (Table I) is adjusted with sodium hydroxide to pH 6.8.

TABLE I.

Composition of Norris medium.

Component	Quantities per liter
Adenine sulphate	17.4 mg
Alanine	200.0 mg
p-Aminobenzoic acid	10.0 mg
Ascorbic acid	2.0 g
Asparagine	100.0 mg
Biotin	5.0 µg
L-Cystine	200.0 mg
Ferrous sulfate ($FeSO_4.7H_2O$)	10.0 mg
Folic acid	10.0 µg
Guanine hydrochloride	12.4 mg
Lactose	35.0 g
Magnesium sulphate ($MgSO_4.7H_2O$)	200.0 mg
Manganese sulphate ($MnSO_4.H_2O$)	6.7 mg
Nicotinic acid	600.0 µg
N-Z case (casein hydrolyzate NBC)	5.0 g
Potassium phosphate (K_2HPO_4)	2.5 g
Pyridoxine hydrochloride	1.2 mg
Riboflavine	200.0 µg
Sodium acetate, anhydrous	25.0 g
Sodium chloride	10.0 mg
Sorbitan monooleate (Tween 80)	0.5 g
Thiamine hydrochloride	200.0 µg
Tryptophan	200.0 mg
Uracil	1.0 mg
Xanthine	10.0 mg
Human milk (defatted)	2 % (vol.)

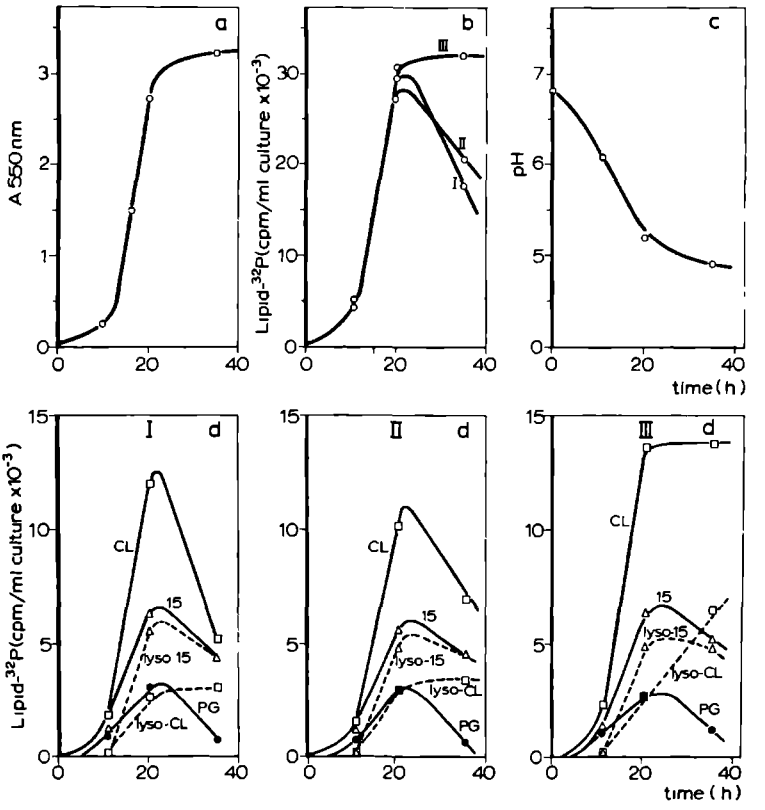

Figure 6.

Efficiency of three lipid extraction procedures related to the growth phase.

B. bifidum var. pennsylvanicus cultivated in the presence of ^{32}Pi is extracted according to three procedures I, II or III.

Procedures: I: Two extractions with acetate buffer (pH 5.0)-methanol-chloroform (6 : 13 : 6, by vol.). II: As I, but followed by two additional extractions with chloroform-methanol (2 : 1, by vol.). III: One extraction with acetate buffer-methanol-chloroform (6 : 13 : 6), followed by three additional extractions with chloroform-methanol (2 : 1).

The combined extracts of each procedure are equalized to a ratio of 6 : 13 : 6 for buffer-methanol-chloroform. For separation into two layers chloroform is added to a ratio of 12 : 13 : 6.

a. Optical density of the culture, b. Lipid radioactivity extracted according to procedures I, II and III, respectively, c. pH of the medium during cultivation, d. Lipid radioactivity of the individual lipids for each procedure. The values of radioactivity are standardized for 1 μ Ci ^{32}Pi added per ml culture.

The added amount of ascorbic acid (2.0 g/l) is twice the concentration given by Norris and the medium is autoclaved after addition of ascorbic acid. Calcium pantothenate, originally prescribed for other strains, is omitted. Instead *Bifidobacterium bifidum* var. *pennsylvanicus* requires pantotheine, which is present in sufficient amounts in caseine-hydrolyzate (N-Z case).

The organism is cultivated anaerobically at 37°C, using a gas phase of nitrogen and carbon dioxide (9 : 1). For batch cultures 5 ml of a 24 h-culture is added per 1 growth medium. To maintain viability the cells are subcultured every two days.

During cultivation growth is monitored turbidimetrically at 550 nm, using a Bausch and Lomb Spectronic 20 spectrophotometer. Appropriate dilutions are made to give an absorbance value of 0.40 - 0.50 at 550 nm (test tubes of 13 mm diameter). The absence of infectants is controlled microscopically and by inoculation of tubes with medium with and without human milk. The number of bacterial doublings (x) in a certain interval is calculated from the equation :

$$\frac{A_2}{A_1} = 2^x \quad .$$

A_1 and A_2 represent the absorbance values at times t_1 and t_2 respectively.

2.2. Radioactive phosphate incorporation.

Incorporation of ^{32}P phosphate into lipids *in vivo* is studied by addition of carrier-free ^{32}P-orthophosphate to the culture in amounts of approximately 1 mCi per liter. In order to enhance the ^{32}P-incorporation the concentration of potassium phosphate in the medium is lowered from 2.5 to 0.25 g/l. Radioactive phosphate is obtained from Philips-Duphar (Petten, Netherlands) in sterile physiological salt solution (1 mCi/ml).

2.3. Extraction of membrane lipids.

A modification of the method according to Bligh and Dyer (1959) has been used for lipid extraction from the bacteria. The extraction system of Bligh and Dyer is a monophasic solution of chloroform-methanol-water. Separation of the homogenous extract into two phases is achieved by addition of chloroform and water. As a result the lower chloroform phase consists essentially of pure chloroform, while the upper layer contains nearly all methanol and water, so that the polar lipids accumulate into the upper layer.

In our experiments 1 g (wet wt) of harvested cells are resuspended in at least 10 ml acetate buffer (0.2 M, pH 5.0). The lipids are extracted by addition of methanol and chloroform yielding a proportion of 6 : 13 : 6 (by vol.) of chloroform-methanol-buffer. After 1 h the cell residue is sedimented and extracted once again. Separation into two layers is achieved by addition of chloroform only, bringing the chloroform-methanol-water ratio to 12 : 13 : 6. The lower layer is collected and the upper layer is extracted once again with the same amount of chloroform to remove all polar lipids. In this way a more complete extraction of the phosphate-containing lipids into the chloroform phase is obtained than by the original Bligh and Dyer method, due to the increased polarity of the lower layer. The chloroform layers are combined and evaporated to dryness under reduced pressure. The lipids are dissolved in chloroform-methanol (1 : 1, by vol.) and stored at -20° C.

The efficiency of the extraction procedure has been determined in the following way. Samples, withdrawn from one culture after three times intervals are divided in three portions each. Lipids have been extracted in three ways from each sample as shown in Fig. 6. The efficiency of the methods I, II and III is similar in the lag phase and late logarithmic phase, but differs markedly after the onset of the stationary phase (fig.6b). Looking at the extraction of the individual lipids (fig.6d I, II, III), it is clear that the differences are in the extraction of cardiolipin (CL) and its lyso-derivatives (Lyso-CL). The decrease in the phosphogalactolipid (compound 15), as well as Lyso-15 and phosphatidylglycerol (PG) during the stationary phase is equal for all three procedures. This is due to a decrease in the cellular amounts of these phospholipids rather than due to incomplete extraction. Procedure I is the method of extraction used in our further studies (Chapters 3, 4 and 5), being the most rapid method for efficient extraction of all phospholipids from cells in the lag phase and in the log phase. An inefficient extraction of the cardiolipins in the stationary phase is revealed by a decline of the extracted lipid radioactivity (Fig. 6). Apparently, cardiolipin is tightly bound, particularly in the stationary phase, which is a common feature for *Bacillaceae* (Lang and Lundgren, 1970; Reaveley and Burge, 1972, p.23).

2.4. Measurement of radioactivity.

Radioactivity is measured with a Packard Tri-Carb liquid scintillation counter model 3380 (New England Nuclear, Dreieichenhain, W.Germany). Lipid samples in chloroform-methanol (1 : 1, by vol.), varying from 10-200 µl, are counted in vials with 10 ml of a solution of Omnifluor (NEN) in toluene (4 g/l). Water-soluble samples up to 200 µl are counted in 10 ml dioxan solution, containing 0.6 g naphtalene, 1.0 ml methanol, 0.2 ml ethyleneglycol and 40 mg Omnifluor (Bray 1960).

^{32}P-labeled compounds, separated by paper chromatography or electrophoresis, are located by autoradiography using Kodak X-ray films (RP/R?) or by scanning with a Berthold thin-layer scanner. Radioactive spots on thin-layer plates are scraped into scintillation vials and counted after mixing with 5 ml water and 10 ml Aquasol (Merck, Darmstadt, W.Germany). Spots developed on paper are cut out and placed in vials with 10 ml scintillation solvent of Bray as given above. Preparatively separated lipids are eluted from silica gel with chloroform-methanol-water (10 : 10 : 1, by vol.). A sample of the eluted lipids is counted as described above.

2.5. Lipid fractionation.

For the study of the phosphogalactolipids from *Bifidobacterium bifidum* var. *pennsylvanicus* it is desirable to remove the neutral lipids and glycolipids present in the total lipid fraction by column chromatography (Vorbeck and Marinetti 1965; Spanner 1973).

A silicic acid column is prepared from a slurry of purified silicic acid, 100 mesh (Mallinckrodt, St. Louis, USA) in chloroform. The total lipid fraction, suspended in chloroform, is placed on the column. The neutral lipids (free fatty acids and glycerides) are not adsorbed by the silicic acid, and are eluted with dry chloroform. Thereafter, the glycolipids are removed with dry acetone. Finally, all phosphate-containing lipids are eluted with methanol and a mixture of chloroform-methanol-water (10 : 10 : 1, by vol.).

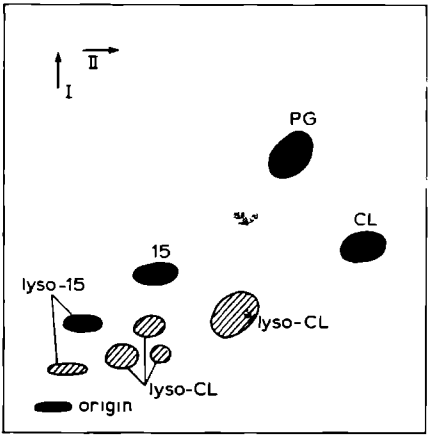

Figure 7.

Thin layer chromatogram of the phosphate-containing lipids.

The chromatogram is developed with chloroform-methanol-7 M ammonia (60 : 35 : 5, by vol.) in the first direction and with chloroform-methanol-acetic acid-water (125 : 37 : 9.5 : 1.5, by vol.) in the second direction.

Abbreviations: PG = phosphatidylglycerol; CL = cardiolipin (diphosphatidylglycerol); 15 = the phosphogalactolipid, described in Chapter 3.

Thin-layer chromatography on silica gel G or HR (Merck, Darmstadt, W.Germany) is used for the separation of the individual phosphate containing lipids. HR plates are used for preparative chromatography, which requires a high degree of purity. Magnesium silicate (3% w/w) is added to silica gel HR as a cement.

A two-dimensional chromatogram is given in Fig. 7. The minor compounds are drawn as hatched spots. The lyso-cardiolipins given in the figure are probably mono- and di-acylcardiolipins. The presence of different spots for one lyso-compound may be due to the presence of a salt form. For instance, the lower spot of lyso-15 migrates in the same way as the higher spot after elution and development on a second chromatogram.

2.6. Alkaline alcoholysis.

Mild alkaline alcoholysis at 37°C (0.025-0.1 M NaOH in methanol or ethanol) is sufficient to split ester linkages of fatty acids (Dawson 1967) leaving the linkages of phosphodiesters, glycosides and alkyl groups intact. The various lipids from an organism can be rapidly characterized by electrophoresis and paper chromatography of the water-soluble deacylation products.

When the identity of a lyso-cardiolipin on thin layer plates is in doubt, certainty is obtained by the method, which also reveals the amounts of contamining lipids.

Deacylation of the lipids is acieved by addition of petroleum-ether (40-60°), ethanol, water and 1 M NaOH in a proportion of 8 : 75 : 6.5 : 2.5 (by vol.), resulting in a final concentration of approximately 0.025 M NaOH.

TABLE II.

Migration by paper electrophoresis and paper chromatography of the deacylation products.

The values for electrophoresis, relative to inorganic phosphate (M Pi), are obtained after 2 h at 30 V/cm in pyridine-acetic acid-water (1 : 10 : 89, by vol.) (pH 3.6) at 0-5 °C. Chromatography is performed at 20 °C during 20 h, using 1-propanol-ammonia-water (6 : 3 : 1, by vol.) as developer.

Lipid	Deacylation product*)	M Pi	Rf
(Lyso)-cardiolipin	GPGPG	0.92	0.33
Phosphatidylglycerol	GPG	0.70	0.46
(Lyso)-compound 15	GPGalG	0.53	0.43

Reference compounds**)	M Pi	Rf
Pi	1.00	0.0
Glycol-P	0.92	0.28
Glycero-3-P	0.84	0.27
Galactose-6-P	0.68	0.09
Galactosylglycerol	0.0	0.59
Galactose	0.0	0.55
Glycerol	0.0	0.67

*) G, P and Gal stand for glycerol, phosphate and galactose.

**) The reference compounds were transformed previously in the acidic form. The salts give considerably lower Rf values.

The polar lipids are first dissolved in a small amount of chloroform-methanol (1 : 1, by vol.). The lipids (10-50 mg) are hydrolyzed in about 10 ml alkaline alcohol by shaking the mixture at 37° C for 15 min. The mixture is then allowed to percolate through a column of Dowex 50 H^+, a cationic resin, suspended in 80% ethanol. The residual non-hydrolyzed lipids are treated once again with the same mixture. The deacylated lipids are eluted with 80% ethanol and with water, the eluate is evaporated to dryness under reduced pressure and the residue is dissolved in a small amount of water. The results obtained by electrophoresis and paper chromatography are given in Table II. Paper sheets used for electrophoresis are Macherey and Nagel no 214or Whatman 3 MM and for chromatography Whatman no 1 or Schleicher and Schuhl no 2045a.

2.7. Staining procedures.

The phosphate-containing lipids separated on silica gel plates are visualized according to Dittmer and Lester (1964). The reagent of Hanes and Isherwood (1949) is used for phosphate staining after paper electrophoresis or paper chromatography. Oxidizable glycerol and galactose moieties

of the lipids and their water-soluble products are stained with periodate-Schiff reagents (Baddiley et al, 1956). A bluish colour often appears with sugars, whereas an α-glycerophosphate moiety yields a purple stain, indicative for the release of formaldehyde. Non-glycosidic sugars are detected with an aniline-hydrogenphthalate spray (Partridge, 1949).

STRUCTURE OF THE PHOSPHOGALACTOLIPIDS

3.1. Introduction

Compound 15 as designation for an unknown phospholipid of *Bifidobacterium bifidum* var. *pennsylvanicus* has been introduced in an earlier

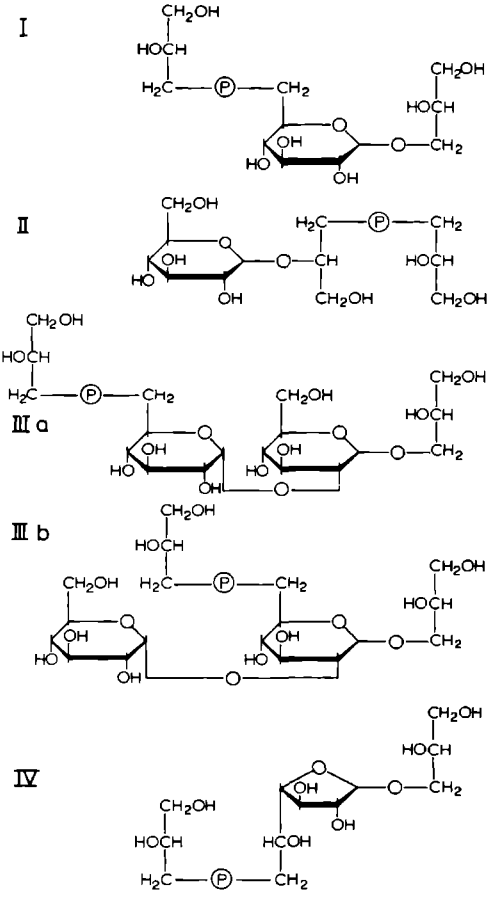

Figure 8.

Deacylated cores of known phosphoglycolipids: I Glycerophosphorylglucopyranosylglycerol (Wilkinson and Bell, 1971); II· Glucopyranosylglycerophosphorylglycerol (Peleg and Tietz, 1973), IIIa and IIIb· Glycerophosphoryldiglucopyranosylglycerol (Ambron and Pieringer, 1971, Smith, 1972, Shaw et al., 1972, Fischer et al., 1973a); IV Glycerophosphorylgalactofuranosylglycerol (Veerkamp and Van Schaik, 1974).

report (Exterkate and Veerkamp, 1969). Preliminary investigations pointed to a cardiolipin-like structure (Exterkate et al., 1971), which proved untenable when galactose was found to be a component of this lipid. Thus, compound-15 has been recognized to belong to the phosphoglycolipids, a new class of lipids containing phosphate, glycerol and a sugar as constituents (Shaw and Stead, 1972).

The core structure of some phosphoglycolipids is illustrated in Fig. 8. Structures I, II and III have been obtained from the literature. The first deacylation product, derived from phosphatidylglucosyldiglyceride, originates from *Pseudomonas diminuta* (Wilkinson and Bell, 1971). Structure I and II are isomers, the latter being obtained from glucosylphosphatidylglycerol, a lipid of a certain halotolerant bacterium (Peleg and Tietz, 1973). Glucosaminyl phosphatidylglycerol is an analogous derivative of phosphatidylglycerol discovered in *Bacillus megaterium* (Op den Kamp et al., 1965). Glycerophosphoryldiglucosylglycerol (IIIa and IIIb) has been found in *Acholeplasma laidlawii*; the structure shown is more plausible than the phosphatidylglucose structure, formerly proposed (Shaw et al., 1972). The same kind of phosphoglycolipid has been isolated from *Streptococcus faecales* and *Streptococcus hemolyticus* (Ambron and Pieringer, 1971 and 1973; Fischer et al., 1973a and 1973b). Structure IV is the core of the phosphogalactolipid of *Bifidobacterium bifidum* var. *pennsylvanicus*, the structure of which is being discussed in this chapter.

3.2. Methods

3.2.1. Isolation of the phosphogalactolipid

Bifidobacterium bifidum var. *pennsylvanicus* is cultivated in 8 1-batches for 16 h and sedimented at 10.000 g. The cells are washed twice with 0.2 M sodium acetate buffer (pH 5.0), centrifuged again and extracted as described in section 2.3. A separate culture of 800 ml is supplied with $^{32}P_i$ (1mCi) and the lipids are extracted in the same manner. The radioactive and non-radioactive total lipids are mixed and fractionated on a silicic acid column (section 2.5). One-dimensional, preparative thin-layer chromatography is applied subsequently to separate the phosphate-containing lipids. The solvent systems chloroform-methanol-acetic acid-water (125 : 37 : 9.5 : 1.5, by vol.) and chloroform-methanol-7 M ammonia (60 : 35 : 5, by vol.) are used sequentially for separation on silicagel G. Finally, the phosphogalactolipids are placed on silicagel HR and developed with chloroform-methanol-water (65 : 25 : 4, by vol.). In this way 1-3 mg of compound-15 could be isolated from 1 litre of culture. The purified lipid is chromatographed two-dimensionally as given in Fig. 7. A single purple spot after periodate-Schiff staining coincides with the ^{32}P-labeled spot detected by autoradiography in pure preparations.

3.2.2. Analytical procedures

The constituent molecules of compound-15 and its deacylation product, isolated by paper electrophoresis (section 2.6), are analyzed as follows.

Phosphorus. Samples containing 100-200 nmoles phosphorus are dried and digested in perchloric acid at 180°C (Bartlett, 1959). Phosphate is determined spectrophotometrically at 830 nm in 2 cm cuvets as the molybdate complex, reduced with 1-amino-2-naphthol-4-sulphonic acid.

Carbohydrate. The method of Radin et al. (1955) is applied, using galactose as a standard. Hexoses in the presence of sulphuric acid form

hydroxymethylfurfural, which gives a colored complex with anthrone. Similar complexes are obtained with pentoses. Dried samples of compound 15 and its deacylation product, containing 300-600 nmol galactose are hydrolyzed for 15 min with 85% phosphoric acid at 90°C and colored by addition of anthrone reagent.

Galactose. Samples containing 100-250 nmoles galactose are hydrolyzed with 2 M HCl for 2 h at 100°C, which is sufficient to hydrolyze phosphodiesters and glycosidic linkages. Galactose is then determined by enzymic oxidation with D-galactose dehydrogenase (EC 1.1.1.48) at pH 8.6 in the presence of NAD^+ yielding NADH in equimolar amounts (Kurz and Wallenfels, 1970). Alkaline phosphatase (EC 3.1.3.1) from *Escherichia coli* is added to the galactose assay medium for enzymic dephosphorylation of galactose phosphate, which is hardly attacked in the 2 h interval with 2 M HCl at 100°C.

Fatty acyl esters. The method of Snyder and Stephens (1959) is applied in which alkaline hydroxylamine reacts with fatty acyl esters to form hydroxamates. The latter give a purple iron-chelate complex in the presence of ferric perchlorate. Samples of 300-900 nmoles are measured at 530 nm in 2 cm cuvets, using methyllinoleate or glyceroltristearate as standard.

Glycerol. This assay is based on the quantitative conversion of glycerol to glycerophosphate with glycerokinase (EC 2.7.1.30) and ATP. ADP, which is formed in equimolar amounts, is regenerated with phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40). The latter reaction is coupled with the conversion of NADH to NAD^+ by lactic dehydrogenase (EC 1.1.1.27) (Garland and Randle, 1962). The decrease in NADH, measured at 340 nm, is equivalent to the original amount of glycerol. Samples containing 100-250 nmoles glycerol are hydrolyzed with 2 M HCl for at least 48 h at 120°C in tubes sealed under vacuum. Strong acid hydrolysis is necessary in order to release all glycerol from glycerophosphate. No significant destruction of glycerol occurs under these conditions. Glycerol is assayed after neutralization with 4 M NaOH.

Sn-Glycerol-3-phosphate. The stereospecificity of glycero-3-phosphate dehydrogenase (EC 1.1.1.8) permits to distinguish between *sn*-glycerol-3-phosphate and *sn*-glycerol-1-phosphate by the conversion of the former to dihydroxyacetone-phosphate in the presence of NAD^+. Since the equilibrium is on the side of *sn*-glycerol 3-phosphate, the reaction is forced to completion by adding high concentrations of the enzyme and NAD^+ and by coupling dihydroxyacetone-phosphate with hydrazine. This results in the formation of NADH in an amount equimolar with the original amount of *sn*-glycerol 3-phosphate (Hohorst, 1970).

A mixture of glycerophosphates is isolated by preparative paper electrophoresis after strong alkaline hydrolysis of the deacylated phosphogalactolipid (1 M NaOH, 2 h, 100°C). From this mixture samples are taken for phosphate and *sn*-glycerol 3-phosphate analysis. *Sn*-glycerol 3-phosphate (Boehringer, Mannheim, W-Germany), serving as a standard, is used in amounts up to 400 nmoles, whereas up to 800 nmoles glycerophosphate mixture is taken.

The enzymes necessary for the various assays are purchased from Boehringer & Söhne, Mannheim, W-Germany.

3.2.3. Alkaline hydrolysis

Deacylated phosphogalactolipid is hydrolyzed with 1 M NaOH for 2 h at 100°C and subsequently freed from sodium ions by treatment with Dowex-50 H^+. The eluate, which is weakly acid due to the presence of phosphate groups, is neutralized with ammonia to prevent degradation of glycosidic linkages. Hydrolysis of phosphodiesters thus results in the formation of monophosphate esters, while glycosidic linkages remain intact.

3.2.4. Oxidation and reduction procedures

The number of vicinal hydroxyl groups is determined from the decrease in absorbance at 225 nm (Dyer, 1956; Kabat and Mayer, 1961). Deacylated lipids or degradation products are oxidized in water, while intact lipids are dissolved in chloroform-ethanol-water (1 : 3 : 1, by vol.). The original $NaIO_4$ concentration is 2 mM in both cases. The initial amount of periodate is about twice as much as required for complete oxidation of the samples. The decrease in optical density, followed for at least 20 h, is multiplied by 10/9, since the absorbance at 225 nm of the resulting iodate is 10% of that of periodate (Kabat and Mayer, 1961).

Formaldehyde, released by periodate oxidation of 1,2-diol groups, is determined by the chromatropic acid reaction (Kabat and Mayer, 1961), using glycerol or serine as a standard.

In order to investigate the position of the substituents of glycerol and galactose, ^{32}P-labeled water-soluble products of the phosphogalactolipids are oxidized with a 5-10 fold excess of 0.05 M $NaIO_4$. The weakly acid periodate solution is previously adjusted to pH 6 with NaOH, when hydrolysis of the labile oxidation products is to be prevented. Ethyleneglycol is added to reduce the excess of periodate and the ^{32}P-labeled oxidation products are reduced overnight with KBH_4 at 4°C. The residual KBH_4 is then destroyed with acetic acid. K^+ and Na^+ ions are removed with Dowex-50 H^+. Borate ions are eliminated by repeated evaporation of the eluate to dryness with methanol (Wilkinson and Bell, 1971). This procedure is also applied for the reduction of galactose phosphate in order to distinguish between galactose-1- and -6-phosphate.

3.2.5. Enzymic deacylation procedure

For the location of the fatty acids in the phosphogalactolipids various lipases have been used: Snake phospholipase A_2 (EC 3.1.1.4) from *Crotalus terrificus terrificus* (5 U/10 µl), phospholipase C (EC 3.1.4.3) from *Bacillus cereus* (1 U/10 µl), a gift from Dr. R. Zwaal (Department of Biochemistry, University of Utrecht), and porc pancreatic lipase (EC 3.1.1.3) (12 U/10 µl), a gift from Dr. R. Verger (Institut de Chimie Biologique, Marseille, France).

The activities of the enzymes (in units per µl) are checked by automatic titration of the released fatty acids (phospholipase A_2) or of the released monophosphate esters (phospholipase C) from egg yolk lipoproteins (Zwaal et al., 1971). Tributirine is used as substrate for lipase (Sémériva et al., 1971). The enzyme reactions are performed in an 8 ml incubation vessel equipped with a magnetic stirrer, a pH electrode (Radiometer 27), connected to a titrator (Radiometer SBR2c), and a supply tube for addition of 0.02 M NaOH.

For the incubation of the phosphogalactolipids with phospholipase A_2 and lipase an emulsion is prepared by sonication for 30 sec with a Bransson sonifier B12. The emulsion contains per ml 1 µmole phosphogalactolipid (30.000 cpm), 50 µmoles Tris-HCl buffer (pH 8.0), 0.4 mg sodium deoxycholate and 2.5 µmoles calcium chloride. The emulsion is divided in 1.2 ml portions. Calcium chloride is raised to 25 µmoles/ml for lipase incubation and in the corresponding blank incubation. The mixture is incubated with 30 U lipase for 2 h at 23°C and with 12.5 U phospholipase A_2 for 2 h at 37°C. During incubation the tubes are vigorously shaken. Then 100 µl 2 M HCl and 4 ml chloroform-methanol (1 : 2, by vol.) are added. Separation in two layers is obtained by adding 1 ml chloroform and 1 ml water. The chloroform layer is collected and the methanol-water layer is once again extracted with 2 ml chloroform.

For phospholipase C incubation the phosphogalactolipids are not emulsified but vigorously shaken with a buffer-ether mixture. 1 µmole phosphogalactolipid (30.000 cpm) is evaporated to dryness in each of three tubes and shaken for 10 min with 1.5 ml ether. The 0.4 ml buffer pH 7.4 is added, containing 40 µmoles Tris-HCl, 4 µmoles calciumchloride and 3 units phospholipase C. Enzyme is added to the blank tube which is previously heated for 10' at 100 °C. The tubes are vigorously shaken for 3 and 5 h extracted as above. Lecithin and phosphatidylglycerol, treated in the same manner, serve as controls.

3.2.6. Physical methods.

Optical rotation is measured with a Perkin-Elmer model 141 polarimeter using a cylindrical cell of 1 ml and 100 mm length. Carbohydrate containing compounds are dissolved in water. Maltose solution, equilibrated overnight, is used as a control.

Gas-liquid chromatography is performed with a Packard model 7821 gas chromatograph. Methylated fatty acids are analyzed on a column of 0.16 inch x 6 ft of 15% diethyleneglycolsuccinate on 60-80 mesh Gas-Chrom P at 168 °C. The methylated fatty acids are prepared by methanolysis of 0.1-5 mg lipid in 0.5 ml hexane with 1 ml BF_3-methanol (10% w/v) at 100 °C for 15 min.

Mass spectrometry, kindly performed by Dr. J.P. Kamerling and Mr. L. Dorland (Department of Organic Chemistry, State University, Utrecht) and by Mr. C. Versluis (Department of Analytical Chemistry, State University, Utrecht), is applied for structural investigation of glycerophosphorylgalactosylglycerol, phosphorylgalactosylglycerol and galactosylglycerol. An A.E.I. MS-902 mass spectrometer is used with the following conditions: ion source temperature 110° - 125°C; electron energy 70 eV; ionizing current 500 µA; accelerating voltage 8 kV. For galactosylglycerol a Jeol JGC-1100/JMS-07 GC-MS combination instrument has been used with the following conditions: ion source temperature 250 °C; electron energy 70 eV; ionizing current 300 µA; accelerating voltage 3 kV; column oven temperature 195 °C; packing material 3% SE-30 on Chromosorb W-AW-DMCS, 80 - 100 mesh). High-resolution mass measurements are performed at a scan speed of 16 sec per mass decade by means of an A.E.I. MS-902 mass spectrometer connected on-line with a Ferranti Argus 500 computer.

Proton magnetic resonance spectroscopy, kindly performed by Dr. J.P. Kamerling (Department of Organic Chemistry, State University, Utrecht) and Dr. J. Bus (Unilever Research Laboratories, Vlaardingen), has been used with galactosylglycerol, employing a 90 MHz Bruker spectrometer with $CDCl_3$ as solvent and tetramethylsilane as internal standard.

In cases of phosphate containing compounds for mass spectrometry the sample is methylated by an ethereal diazomethane solution before silylation. One µmole phosphate-containing product is dissolved in 75 µl dimethylsulfoxide (DMSO) and diazomethane-ether is added for the esterification till the solution becomes yellow. Then the excess of diazomethane is removed by evaporation. The DMSO is evaporated by means of a mercury diffusion pump. Subsequently, the product is silylated with pyridine-hexamethyldisilazane-trimethylchlorosilane (10 : 2 : 1, by vol.) at 23 °C.

Similarly, hydroxyl-containing compounds are silylated for analysis by gas chromatography (glycol, threitol, galactosylglycerol) and for proton magnetic resonance spectroscopy (galactosylglycerol).

Methylated fatty acids are prepared for gas chromatography by methanolysis of 0.1-5 mg lipid in 0.5 ml hexane with 1 ml BF_3-methanol (10% w/v) at 100 °C for 15 min.

TABLE III.

Composition of compound-15 and its deacylation product.

Molar amounts relative to phosphate are given as means for 6 preparations of compound-15 and for 5 preparations of the water-soluble product. Glycerol is determined in all preparations, galactose and acyl groups in 2 preparations.

	Compound-15	Deacylation product
Phosphate	1.00	1.00
Galactose*)	0.54	0.53 (0.87)
Hexose	0.88	1.05
Glycerol	2.05	1.90
Acyl groups	1.88	-

*) The galactose assay is performed after acid hydrolysis.
The value is increased by subsequent addition of alkaline fosfatase from *E.coli* to the value in parentheses.

3.3. Results

3.3.1. Preliminary investigations.

Separation of the extracted ^{32}P-labeled lipids on thin layer plates, followed by autoradiography, shows that compound-15 is a phosphate-containing lipid by autoradiography (Fig. 7). Staining with periodate-Schiff reagent reveals a purple stain, indicative for the presence of 1.2-diol groups. Contaminating glycolipids present on the chromatogram give a bluish colour. Trigalactosyldiglyceride, which has about the same polarity as compound-15, is not completely removed from the phospholipid fraction after column chromatography. Any contamination of galactose from glycolipids has been excluded in the deacylated lipid by preparative electrophoresis. Galactose and glycerol have been shown to be constituents of the deacylation product of compound 15 after acid hydrolysis, which proves compound-15 to be a phosphogalactolipid. The absence of amino groups is proved by the absence of staining with ninhydrin of the intact lipid as well as of the deacylated compound. The presence of an aminogroup, beside the phosphate group, would also give the deacylated compound a zwitterionic character, which is in disagreement with its anodic migration in electrophoresis. The presence of sulphate could be excluded by the lack of ^{35}S-incorporation into the lipids after addition of ^{35}S-sulphate to the growth medium.

3.3.2. Structure of the deacylated core.

Acid hydrolysis of deacylated compound 15 (2 M HCl, 2 h, 100 ^{o}C) releases D-galactose as is demonstrated by the enzymic assay. Its recovery, however, is less than the total carbohydrate content (Table III), determined according to the method of Radin (section 3.2.2.).
The proportion of galactose in the enzymic assay is raised after addition of alkaline phosphatase from 0.53 to 0.87 relative to the phosphate content. This indicates that i) D-galactose is the only carbohydrate present and

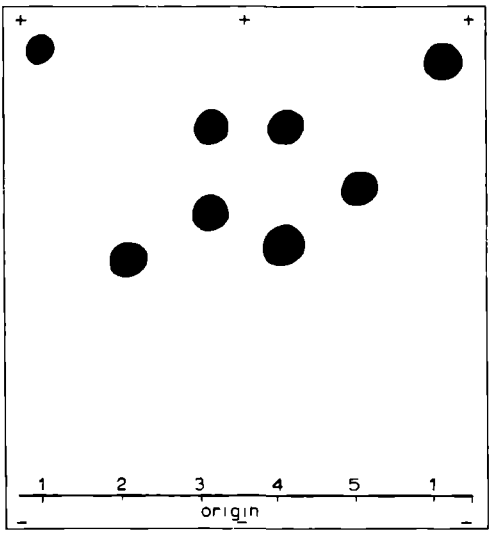

Figure 9.

Electrophoresis of phosphate containing products of acid and alkaline hydrolysis. 1 Inorganic phosphate, 2 Deacylated phosphogalactolipid, 3 Acid hydrolyzate of the deacylated phosphogalactolipid (2M HCl, 2 h, 100°C), 4 Alkaline hydrolyzate of the deacylated phosphogalactolipid (1 M NaOH, 2 h, 100°C), 5 Deacylated phosphatidylglycerol.

TABLE IV.

Alkaline and acid hydrolysis of the deacylation product of compound-15.

The conditions of hydrolysis are 1 M NaOH, 2 h, 100 °C and 2 M HCl, 2 h, 100 °C. ^{32}P-labeled products are separated by electrophoresis. GPG, prepared from the same organism is treated similarly Glycerol and galactosylglycerol, released on alkaline hydrolysis of the deacylation product, are eluted from the origin and are found in a molar ratio of 1 00 1 12, respectively. Galactosylglycerol is calculated as the difference of total and free glycerol present in the eluate.

Compound	Hydrolysis	^{32}P-labeled product	% radioactivity	MPi
GPGalG	alkaline	GP	53	0.82
		PGalG	46	0.56
		Pi	1	1 00
GPGalG	acid	GP	55	0.82
		GalP	35	0.68
		Pi	10	1.00
GPG	alkaline	GP	99	0.82
		Pi	1	1 00
GPG	acid	GP	96	0.80
		Pi	4	1.00

G stands for glycerol, P for phosphate, Gal for galactose. MPi is mobility relative to that of inorganic phosphate

ii) galactose and galactose phosphate are released in approximately equimolar amounts on acid hydrolysis. Enzymic dephosphorylation of the monophosphate esters is preferable to prolonged acid hydrolysis, since galactosephosphate is rather resistant and the released galactose decomposes during the latter treatment. This is shown by control experiments in which the galactose recovery from galactose and galactose-6-phosphate after 10 h of acid hydrolysis is found to be 61% and 52%, respectively.

No inorganic phosphate is released upon incubation of the deacylated lipid with alkaline phosphatase. This finding, combined with the occurrence of glycerophosphate and galactosephosphate on acid hydrolysis (Fig. 9, Table IV) points to a phosphodiester linkage between glycerol and galactose. The molar ratio for phosphate-glycerol-galactose of 1 : 2 : 1 (Table I) suggests that the second glycerol moiety is bound to galactose through a glycosidic linkage. This linkage is alkali-stable. Galactosyl-glycerol has indeed been demonstrated after hydrolysis with 1 M NaOH (2 h, 100 ^{o}C) by gas chromatography and mass spectrometry. Furthermore, a phosphate-containing fragment (MPi 0.56) is released (Fig. 9, Table IV), yielding galactosylglycerol (GalG) after dephosphorylation with alkaline phosphatase. Therefore, the fragment of MPi 0.56 must be phosphorylgalactosyl glycerol. These findings agree with the structure of glycerophosphoryl-galactosyl-glycerol (GPGalG) for the core of the deacylated phosphogalactolipid.

3.3.3. Location of the phosphate moiety.

^{32}P-labeled deacylated lipid (GPGalG) is subjected to acid hydrolysis (2 M HCl, 2 h, 100 ^{o}C). Subsequently, preparative electrophoresis is applied to separate the fragments. Galactose phosphate is detected by autoradiography (Fig. 9). Its mobility relative to inorganic phosphate (MPi) is 0.68, corresponding to that of hexose 6-phosphates used as reference compounds. Cyclic intermediate formation is known to occur upon acid hydrolysis of phosphodiesters (Strickland, 1973), similarly to what happens during base hydrolysis. This means that the phosphate linked to galactose can partly migrate to the adjacent hydroxyl group after breakage of the phosphodiester bond (Fig. 10). This effect makes it difficult to establish the original phosphate position in the molecule. The occurrence of galactosylglycerol after alkaline hydrolysis excludes the location of phosphate at carbon atom 1, which is also in agreement with the resistance of the galactose phosphate product to acid hydrolysis. Furthermore, the absence of a glycosidic linkage in galactose phosphate is demonstrated by the staining with anilinehydrogenphthalata and by its reduction with borohydride. Addition of potassium borohydride to galactose phosphate and to the deacylated lipid, prior to the enzymic determination of galactose in the presence of alkaline phosphatase, yields a proportion of phosphate to galactose of 1.0 : 0.08 and 1.0 : 0.87, respectively, indicating that only the galactosephosphate fragment is reduced.

Periodate oxidation of galactose phosphate originating from compound 15, followed by reduction with borohydride, results in the formation of glycol phosphate (MPi 0.90), glycerophosphate MPi (0.80) and inorganic phosphate in a proportion of 49 : 45 : 6. Glycol phosphate originates from galactose-6-phosphate. Galactose-5-phosphate, which may have been formed by phosphate migration can explain the occurrence of glycerophosphate.

Carbon atom 6 of galactose could be established as the original location of the phosphate group in compound-15 by a mild acid hydrolysis of the deacylation product. A phosphate containing fragment has been isolated by

preparative electrophoresis (MPi 0.62). Analysis of this product gives a molar ratio of phosphate, glycerol, and periodate required for oxidation of 1.0 : 1.19 : 5.02, consistent with the structure of glycero-phosphoryl-galactose, with phosphate located at carbon atom 6 of galactose. This is confirmed by the finding that periodate oxidation of this product and subsequent reduction and acid hydrolysis yielded only ethyleneglycol phosphate, but not glycerophosphate.

3.3.4. Configuration of the galactose moiety.

The deacylation product of compound-15, isolated by electrophoresis, has been subjected to strong alkaline hydrolysis (section 3.2.3). After paper chromatography in solvent system 1-butanol-pyridine-water (6 : 4 : 3, by vol.) two spots cochromatographed with galactofuranosylglycerol and galactopyranosylglycerol used as reference compounds (Rf 0.44 and 0.29, respectively).

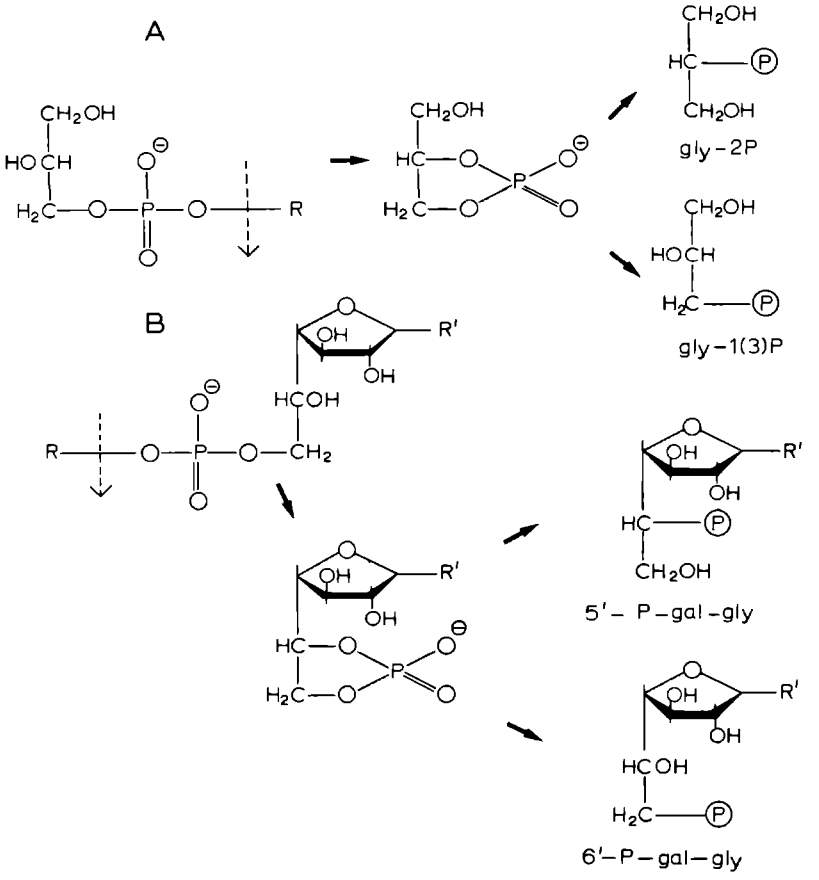

Figure 10.

Phosphate migration on alkaline hydrolysis of the phosphodiester linkage

The reference compounds originated from monogalactosyldiglycerides of spinach and *B.bifidum* var.*pennsylvanicus*. After elution galactose has been recovered in both compounds (Rf 0.44 and 0.29) in a ratio of 81 : 19, respectively, when assayed with galactosedehydrogenase (see 3.2.2).

The products of strong alkaline hydrolysis have also been analyzed by gas chromatography (see 3.2.6). Two compounds were found with retention times identical to those of galactofuranosylglycerol and galactopyranosylglycerol almost in the same proportion as given above.

The mass spectrum (MS-902) of the trimethylsilyl derivative of the isolated galactofuranosylglycerol (Rf 0.44) establishes its furanose configuration by the high ratio of the peaks m/e 217 and m/e 204 (217/204 = 19.8) and of the peaks m/e 205 and m/e 204 (205/204 = 1.8). The ratio 217/204 in the mass spectrum (Jeol GC-MS combination) of the galactopyranosylglycerol preparation (Rf 0.29 in 1-butanol-pyridine-water 6:4:3) amounts to only 0.25, which confirms the presence of the pyranose form in this preparation. (See for comparison the values published by Kärkkäinen (1969) and Vilkas et al. (1973)). The furanose isomer present as contamination in the galactopyranosylglycerol preparation is removed by gas chromatography preceding to mass spectrometry.

The mass spectrum of the deacylated phosphogalactolipid establishes a molecular weight of 926 for the compound. Furthermore, it can be deduced from the mass spectrum that one glycerol unit is coupled to the anomeric carbon atom of galactose, that one glycerol unit is coupled to phosphate and that the galactose unit occurs mainly in the furanose form (217/204 = 4.5) (Dorland et al, to be published).

The furanose configuration in the isolated phosphorylgalactosylglycerol fragment is similarly demonstrated by mass spectrometry, because a value of 2.0 is found for the ratio 217/204. Other indications for the presence of a furanose form are the presence of m/e 227 ($C_3H_7O_4P$(OTMS)) and the absence of m/e 241 (see Table V). In pyranose ring structures, containing a phosphate group at C-6, m/e 227 is absent, while m/e 241 ($C_4H_9O_4P$(OTMS)) is present (see for comparison the data published by Zinbo et al. (1970) for completely silylated components).

The location of the phosphate moiety at carbon atom 5 and 6 of galactose (indicated in Fig. 10) could not be distinguished on account of the mass spectrum. Typical ionic fragment of the mass spectra are characterized (Table V). E.g. the fragments m/e 204 and m/e 205 contain two adjacent carbon atoms, each bearing a trimethylsilyl group. Peak m/e 227 is an analogon of peak m/e 343 found by Zinbo and Sherman (1970) in the mass spectrum of galactofuranoside 6-phosphate. Peak m/e 227 corresponds to the fragment consisting of dimethylphosphate which is probably linked to carbon atom 3 or 4 of galactose after migration during fragmentation. The peak m/e 337 occurs in all spectra and corresponds to the glycerol moiety connected to carbon atom 1 of galactose.

The glycosidic linkage of the galactofuranosylglycerol preparation has a β-configuration, which appears from its optical ration $[\alpha]_D$ of -102^o. Plackett (1967) reported for a β-D-galactofuranosyl glycerol preparation an $[\alpha]_D$ value of -80 ± 13, Reeves et al. (1964) a value of -73^o. Reported values for the pyranose analogue vary between +7.5 and $+8.1^o$ (see Veerkamp, 1972). The β-configuration of the furanose isomer has also been established by proton magnetic resonance spectroscopy on account of a coupling constant $J_{1,2}$ of 0 Hz of the H_1-atom at δ = 4.80 ppm. The optical rotation of glycerophosphorylgalactosylglycerol amounts -46^o. The negative value is reduced in comparison to that of galactofuranosylglycerol by the presence of the glycerophosphate moiety and by contamination with the pyranose isomer.

Glycerophosphorylgalactosylglycerol is also treated with periodate in order to investigate the contribution of the pyranose isomer to this preparation. Subsequently, the oxidation product of the deacylated compound-15 is reduced with borohydride and hydrolyzed with 2 M HCl.
The pyranose form will yield glycerophosphate instead of threitolphosphate (Fig. 11) as a consequence of an additional oxidation between carbon atoms 3 and 4 of galactose. Both compounds move with different rates on paper electrophoresis on account of the difference in molecular weight, glycerophosphate with MPi of 0.84, threitolphosphate with MPi of 0.72. The phosphate containing product with MPi of 0.72 is indeed recovered, which is in agreement with the furanose configuration (Table VI). No glycerophosphate could be demonstrated, indicating that the pyranose configuration can account for only a very small fraction. Hydrolysis of the phosphodiester bond between glycol and threitol at either side of the phosphate moiety will give glycol and threitolphosphate in equimolecular amounts just as threitol and

TABLE V.

Characteristics of ionic fragments in mass spectrometry of deacylated compound 15 and its galactose containing products on alkaline hydrolysis.

Galactosylglycerol (GalG) and phosphorylgalactosylglycerol (PGalG) are alkaline hydrolysis products (1 M NaOH, 2 h, 100 °C) of the deacylated phosphogalactolipid (GPGalG). All preparations are isolated by electrophoresis, followed by paper chromatography. Gal(f) and Gal (p) stand for the furanosyl- and pyranosyl configurations of galactose. M and TMS stand for methyl- and trimethylsilyl groups, respectively.

Compound	m/e	Ionic fragment
Gal(f)G	686	M^+
	671	M^+ minus $\cdot CH_3$
	451	M^+ minus $\cdot OCH_2$-CHOTMS-CH_2OTMS
	337	TMSO-CH=$\overset{+}{O}$-CH_2-CHOTMS-CH_2OTMS
	217	TMSOCH=CH-CH=$\overset{+}{O}$TMS
	205	CH_2OTMS-CH=$\overset{+}{O}$TMS
	204	TMSO$\dot{C}$H-CH=$\overset{+}{O}$TMS
Gal(p)G	686	M^+
	671	M^+ minus $\cdot CH_3$
	451	M^+ minus $\cdot OCH_2$-CHOTMS-CH_2OTMS
	337	TMSO-CH=$\overset{+}{O}$-CH_2-CHOTMS-CH_2OTMS
	217	TMSOCH=CH-CH=$\overset{+}{O}$TMS
	204	TMSO$\dot{C}$H-CH=$\overset{+}{O}$TMS
PGal(f)G	722	M^+
	707	M^+ minus $\cdot CH_3$
	487	M^+ minus $\cdot OCH_2$-CHOTMS-CH_2OTMS
	337	TMSO-CH=$\overset{+}{O}$-CH_2-CHOTMS-CH_2OTMS
	227	$(CH_3O)_2$PO-$\overset{+}{O}$=CHOTMS
	217	TMSOCH=CH-CH=$\overset{+}{O}$TMS
	204	TMSO$\dot{C}$H-CH=$\overset{+}{O}$TMS
GPGal(f)G	911	M^+ minus $\cdot CH_3$
	691	M^+ minus $\cdot OCH_2$-CHOTMS-CH_2OTMS
	475	P$(OCH_3)(OTMS)_2$-OCH_2-CHOTMS-$CH_2O\overset{+}{T}MS$
	337	TMSO-CH=$\overset{+}{O}$-CH_2-CHOTMS-CH_2OTMS
	217	TMSOCH=CH-CH=$\overset{+}{O}$TMS
	204	TMSO$\dot{C}$H-CH=$\overset{+}{O}$TMS

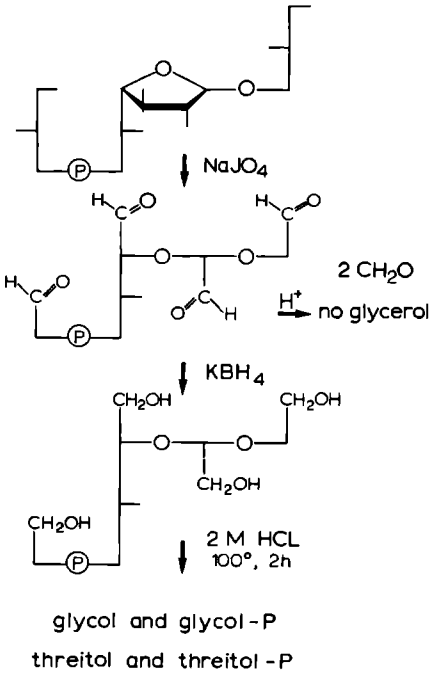

Figure 11.

Degradation by periodate oxidation of the deacylated phosphogalactolipid.

TABLE VI.

Phosphate containing products of periodate oxidation followed by reduction derived from compound-15. Electrophoretic and chromatographic characteristics.

The products are separated by paper electrophoresis in pyridine-acetic acid-water (1 : 10 : 89) (pH 3.6) and by paper chromatography using 1-propanol-ammonia-water (6 : 3 : 1) as developer. The spots are detected by autoradiography.

	MPi	Rf
GPGalG	0.53	0 43
GPGal ox,red	0.68	0.49
*) GPGal ox,red,H^+	0.72	0.20
	0.91	0.27
GPG	0.70	0.46
GPG ox,red	0.81	0.54
GP	0.84	0.27
Glycol-P	0.92	0.28

*) Acid hydrolysis is applied after oxidation and reduction.
Glycerol, phosphate and galactose groups are designated as G, P and Gal, respectively.

glycolphosphate (Fig. 11). Gas chromatographic analysis (see 3.2.6) demonstrated also two peaks corresponding to glycol and threitol, but not to glycerol.

3.3.5. Structure of the glycerophosphate moiety.

Periodate consumption for the oxidation of the deacylated lipid amounts to 3 moles $NaIO_4$ per mole phosphate. A simultaneous release of 2 moles formaldehyde indicates that both glycerols in the deacylated lipid are oxidized, whereas 1 mole $NaIO_4$ is used for the breakage of the ring structure (Fig. 11). After hydrolysis of the oxidation product no glycerol is recovered, confirming that both glycerols are substituted at one α-carbon atom only. Thus, the glycerophosphate moiety can have the *sn*-1 or *sn*-3 stereoconfiguration. Although both isomers can be distinguished with the stereospecific enzyme glycerol-3-phosphate dehydrogenase, we must bear in mind that phosphate migration occurs during hydrolysis of the phosphodiester linkage (Fig. 10). After alkaline hydrolysis 55% of the phosphate is found to have migrated to the adjacent hydroxyl, which has been monitored after periodate oxidation (Table VII). Less than 3% is recovered with glycerol-3-phosphate dehydrogenase, indicating that the deacylated lipid has the *sn*-glycerol 1-phosphate configuration. Glycerophosphorylglycerol, prepared by deacylation of phosphatidylglycerol from the same organism is used as a reference compound. After alkaline hydrolysis of this compound glycerophosphate contains 99% of the original phosphate (Table II). The values for the different configurations in the glycerophosphate mixture agree very well with the meso-diester *sn*-glycero-3-phosphoryl-1'-*sn*-glycerol as the original structure of this compound.

3.3.6. Structure of the diglyceride moiety.

The intact phosphogalactolipid (compound-15) has been shown to contain two fatty acids by the hydroxamate method and by gas chromatography using nonadecanoic acid as an internal standard. The presence of alkyl-ether linked fatty acids, characteristic for plasmalogens (Goldfine, 1972), has been excluded, because these are known to be resistant to alkaline alcoholysis. Periodate oxidation of the intact lipid releases one molecule formaldehyde indicating that only one glycerol is oxidized in the intact lipid (Table VIII). Apparently, the ring structure of galactose remains intact, because the periodate consumption is equivalent to the amount required for the release of one molecule formaldehyde. Therefore, it could be that galactose carries one fatty acid at carbon atom 2 or 3. Investigation of lyso-compound 15, which has only one fatty acid, was thought to give information about the presence of an acyl group on the sugar moiety. A periodate consumption of two moles is expected, when only one fatty acid would be linked either with glycerol or with galactose, in which case formation of one or two moles formaldehyde, respectively, would occur. Lyso-compound 15 has therefore been isolated from the same organism. Analysis of this lipid (Table VIII) suggests that the sugar moiety is resistant to oxidation just as in compound-15, because one monoacylated glycerol is not oxidized. The furanose configuration of compound-15 can only be attacked by periodate between carbon atom 2 and 3. The attack is hampered by the trans-position of the hydroxyls. Although the deacylated lipid is oxidized in aqueous solution, this does not occur, apparently, in the organic system in which the lipids are dissolved. Furthermore, a steric hindrance caused by the

TABLE VII.

Analysis of the glycerophosphates liberated or strong alkaline hydrolysis of deacylated compound-15.

Hydrolysis condition 1 M NaOH, 2 h, 100 °C The isomers of the liberated glycerophosphates are given as relative amounts. α and β glycerophosphates are determined after periodate oxidation and reduction by counting the ^{32}P-activity of glycolphosphate and glycerolphosphate. The values of *sn*-glycerol 1-phosphate are calculated as the difference between α-glycerophosphate and *sn*-glycerol-3-phosphate.

	βGP	αGP	sn-G-1-P	sn-G-3-P
GPGalG	55	45	42	3
GPG	56	44	24	20

TABLE VIII.

Quantitative results of periodate oxidation of compound-15 and its derivatives

Periodate consumption and formaldehyde production are given as molar ratios to phosphate Deacylated cardiolipin, used as a control, is prepared from the same organism.

	P	Used $NaIO_4$	Formaldehyde
Deacylated compound-15	1.00	3.35	2.03
Lyso "	1.00	1.23	0.74
Intact "	1.00	1.00	0.71
Deacylated cardiolipin	2.00	1.86	1.76

fatty acid chains may also affect the resistance of the lipid galactose towards oxidation (Garner et al, 1958).

In order to determine which glycerol carries both fatty acids, compound 15 has been incubated with the phospholipases A_2 and C and with lipase. Enzymic deacylation is only observed with lipase (Table IX), indicating the presence of a diglyceride moiety. When the glycerophosphate unit would be acylated, giving rise to a 1-*sn* phosphatidyl group, enzymic deacylation would be observed with phospholipase C, but not with phospholipase A_2, which requires a 3-*sn*-phosphatidyl moiety. The partition of ^{32}P between the aqueous and organic phases during extraction after the enzyme incubation varies for emulsions prepared by sonication. However, using a control in each experiment, enzymic hydrolysis of the fatty acid esters can be distinguished from mechanical or chemical deacylation.

The fatty acid composition of compound-15 has been determined by gas chromatography of the methyl esters. The main fatty acids are 16 : 0, 18 : 0 and 18 : 1, amounting to 33%, 20% and 29%, respectively.

TABLE IX.

Action of phospholipases and lipase on compound-15 and its lyso-derivative.

After enzymic incubation of emulsions of the ^{32}P-labeled substances chloroform and methanol are added and the aqueous and organic phases are allowed to separate. The partition of ^{32}P-activity over the aqueous- and organic phases is determined. Thin-layer chromatography of the lipids in the organic phase is applied to determine the relative amounts of compound-15 and its lyso-derivative.

Substance	Enzyme	% ^{32}P-activity		Ratio
		aqueous phase	organic phase	Compound-15:lyso-compound-15
Compound-15	-	26	74	91 : 9
"	Phospholipase A_2	27	73	92 : 8
"	-	10	90	78 : 21
"	Lipase	28	72	18 : 79
"	-	6	94	ND*) ND
"	Phospholipase C	9	91	" "
Lyso-compound-15	-	5	95	" "
"	Lipase	64	36	" "

*) ND stands for not determined.

3.4. Discussion and conclusions.

Summarizing the results, the structure of the galactofuranose containing lipid, designated as compound-15 is 1(3)-O-[6'-(*sn*-glycero-1-phosphoryl) β-D-galactofuranosyl]-diglyceride. Structure IV in Fig. 8 represents its deacylation product, but takes no notice of the stereoconfiguration of the glycerophosphate moiety and of the glycosidic linkage. The presence of a *sn*-1,2 diglyceride is very likely, being the most abundant configuration in glycolipids from natural sources (Brundish et al, 1965; Shaw, 1970), but no experimental evidence has been obtained. Therefore, the notation 1(3)-diglyceride is used. A glycosidic linkage at carbon atom 2 of glycerol is excluded, because both glycerols in the deacylation product can be oxidized with periodate. Hitherto the presence of *sn*-glycerol 1-phosphate in phosphoglycolipids has been reported only for glycerophosphoryldiglucosyl diglyceride from *Streptococci* (Fischer et al, 1973a).

Phosphoglycolipids containing galactose have not been described previously. The furanosyl structure of the sugar moiety is also unique for this new class of lipids. The coexistence of the pyranose form is recognized from the fragments galactosylglycerol and phosphorylgalactosylglycerol by chromatography. Gas chromatographic analysis reveals that the galactosylglycerol fragment occurs for 14% of the pyranose configuration. The discovery of the pyranose configuration in the phosphorylgalactosylglycerol fragment, upsets the first assumption that this compound could only be formed through a cyclic phosphodiester between carbon atom 5 or 6. Phosphoglycolipids containing glucose in the pyranose form do not yield any detectable product in which the phosphate-glucose linkage is retained upon alkaline hydrolysis (Fischer, 1970; Ambron and Pieringer, 1971; Shaw et al, 1972).

Galactofuranosyl isomers can easily give a cyclic phosphodiester intermediate between carbon atom 5 and 6 of galactose. In addition, the orientation of the hydroxyl at carbon atom 4 of the galactopyranose isomer seems to favour a phosphodiester intermediate between carbon atom 4 and 6. Probably the orientation of the hydroxyl at carbon atom 4 of the galactopyranose isomer also favours an intermediate phosphodiester with carbon atom 6 of galactose but not of glucose. The cis-trans orientation of the neighbouring hydroxyl groups markedly influences the products of alkaline hydrolysis, as has been established by Brown et al (1958). The galactopyranose structure will be preferentially oxidized with periodate between carbon atom 3 and 4 owing to the cis-orientation of the hydroxyls. Therefore, it is unlikely that threitol-phosphate could be formed by incomplete oxidation of the pyranose structure and subsequent acid hydrolysis. The galactosyldiglycerides in *B.bifidum* var. *pennsylvanicus* have been found in both configurations (Veerkamp, 1972). Galactofuranosides have also been detected in the lipids of *Bacteroides symbiosus* (Reeves et al, 1964, *Mycoplasma mycoides* (Plackett, 1967), *Flavobacterium thermophilum* (Oshima and Yamakawa, 1972). Galactofuranose has been found as a constituent of an arabinogalactan in *Mycobacterium tuberculosis* (Vilkas et al, 1973).

The presence of galactofuranosyldiglyceride in *B.bifidum* var.*pennsylvanicus* suggests its precursor function for the biosynthesis of compound-15. The glycerophosphate moiety probably originates from phosphatidylglycerol as will be demonstrated in chapter 5.

The absence of a fatty acid linked to galactose in compound-15 is suggested by the periodate oxidation of compound-15 and its lyso-derivative, but the results do not yield conclusive evidence. It is therefore noteworthy that both galactofuranosyldiglycerides from this organism have been described as acylated galactose compounds (Veerkamp, 1972). Evidence for the existence of a lipid with more acyl groups than compound-15 has also been obtained (Veerkamp and Van Schaik, 1974). A more apolar lipid, amounting to less than 1% of the total lipid phosphorus, has been isolated from *B.bifidum* var.*pennsylvanicus*. Its deacylation product shows the same electrophoretic behaviour as that of compound15 Glycerophosphate and galactosylglycerol are also found as products of strong alkaline hydrolysis. The latter compound cochromatographed with galactofuranosylglycerol and gave a positive galactose assay after acid hydrolysis Only 5% of the released glycerophosphate is recovered as *sn*-glycerol-3-phosphate, suggesting the presence of *sn*-glycerol-1-phosphate in the intact lipid as found in compound 15.

Phosphoglycolipids from *Lactobacilli* may be covalently bound with lipoteichoic acids (Wicken and Knox, 1970, Shaw and Stead, 1972). Teichoic acids, resembling the structure of deacylated compound-15, are the polygalactosylglycerolphosphates from *Bacillus licheniformis* ATCC (Burger and Glaser, 1966), and from lactic *Streptococci* (Elliott, 1963). From our present knowledge of *B.bifidum* var.*pennsylvanicus* it is impossible to say that lipoteichoic acids would be anchored to the membrane through the phosphogalactolipids. A metabolic interrelationship between phosphatidylglycerol and the phosphogalactolipids is suggested by some experiments described in chapter 5. Phosphatidylglycerol is also known to be a precursor of cardiolipin. In the next chapter the biosynthesis of the phosphogalactolipid and its lyso-derivative will be compared with that of the other phospholipids present under various conditions.

THE INFLUENCE OF SOME GROWTH CONDITIONS ON THE MEMBRANE PHOSPHOLIPID COMPOSITION

4.1. Introduction

In the previous chapter the membrane lipid compound 15 is characterized and its structure is elucidated. Other phosphogalactolipids, namely its lyso- and acylated derivatives are also characterized, though the configuration of the galactose moiety is less extensively investigated.

In this chapter we want to consider the phosphogalactolipids in relation to the other phospholipids of the cell membrane. A previous study (Exterkate et al, 1970) of membrane preparations of *Bifidobacterium bifidum* var. *pennsylvanicus* reports that protein, lipid, lipid-phosphorus and lipid-galactose accounts for 70, 7.8, 0.10 and 0.61% respectively of the dry weight of the material. After cell wall inhibition by lack of human milk the amounts of protein and lipid-phosphorus do not change, while the total lipid content is increased somewhat (to 9.5%) and lipid-galactose is decreased (to 0.39%). We obtained all information about lipid-phosphorus and lipid-hexose from extracts of whole cells.

In this chapter we shall describe the composition of the phospho(galacto)-lipids of *Bifidobacterium bifidum* var. *pennsylvanicus* for growth under different environmental conditions. The organism is cultivated in the Norris medium (Table I) with and without human milk, while the effects of pH, temperature and inhibition by antibiotics are also studied.

4.1.1. Phenotypic variations of the cell periphery

Micro-organisms are dependent upon the environment for supplies of biosynthetic raw materials and energy, just as plants and animals. Some bacteria are extraordinarily adaptable in that they can use an enormous variety of substrates for growth. Chemical and physical environmental factors, which affects the activities of micro-organisms, have been extensively described (Rose 1970).

Adaptation in order to maintain the viability and functioning of the organism may result in changes of the composition of the cell wall (Ellwood and Tempest 1972) and cell membrane (Goldfine 1972). E.g., when *Bacillus subtilis* var. *niger* is limited by phosphate starvation, it stops the synthesis of cell wall teichoic acid and replaces it by teichuronic acid, a phosphate-free anionic polymer of glucuronic acid and N-acetylgalactosamine (see Ellwood and Tempest, 1972). Similarly, changes in the aminoacid composition of the growth medium of *Staphylococcus aureus* may cause substantial changes in the peptide "bridges" in peptidoglycan (Schleifer et al, 1969).

Such phenotypic variations have also been reported for the membrane of *Bacillus subtilis* (Bishop et al; 1967; Minnikin et al; 1972). Progression from the exponential to the stationary phase is accompanied with a decrease of the RNA content in isolated membranes and phosphatidylethanolamine is replaced by diglucosyldiglyceride.

4.1.2. Mode of action of antibiotics

The synthesis and composition of the membrane phospholipids are studied after cell wall inhibition caused by lack of human milk or by the action of antibiotics. Similarly, the effect of chloramphenicol, streptomycin and actinomycin D on the phospholipids is also investigated.

Antibiotics are defined as chemical compounds produced by micro-organisms, which in low concentration inhibit growth and activity of other micro-organisms. The specificity and the site of action of different antibiotics have been described in various reviews (see Bücher and Sies, 1969; Hochster et al; 1972; Salton and Tomasz; 1974).

Penicillin, D-cycloserine, vancomycin and bacitracin are inhibitors of bacterial cell wall synthesis. Penicillin interferes with the cross-linking reaction of the linear peptidoglycan strands, which occurs outside of the cell membrane. This antibiotic appears to mimic the conformation of the D-alanine-D-alanine moiety of the cell wall nucleotide-precursor. Vancomycin and bacitracin inhibit the polymerization of the cell wall nucleotides to linear peptidoglycan strands. The wall precursors, synthetised in the cytoplasm, are translocated across the membrane by a lipid carrier. This carrier is now known to be a C55-isoprenoid alcohol linked to the nucleotide through a pyrophosphate linkage (Lennarz and Scher, 1972). Vancomycin inhibits the transfer of wall precursor from the carrier to the growing strand. Bacitracin blocks the regeneration of the lipid carrier, and also changes the integrity of the cytoplasmic membrane. D-cycloserine inhibits the synthesis of the cell wall nucleotide, which means that its transport across the membrane is a prerequisite for its action. By its structural similarity to D-alanine it acts as a metabolic antagonist for the enzymes alanine racemase and D-ala-D-ala synthetase.

Chloramphenicol and streptomycin are inhibitors of protein synthesis by affecting the 70 S type ribosomes of bacteria. The 50 S subunit of the bacterial ribosome is the site of action for chloramphenicol, inhibiting possibly the peptide-bond formation. Streptomycin blocks the initiation of the polypeptide synthesis, possibly by inhibition of the translocation of formyl methionyl-tRNA or of peptidyl-tRNA from the acceptor to the donor site of the ribosome.

Actinomycin-D inhibits protein synthesis in bacteria as well as in eucaryotic cells at the level of transcription. By its interaction with template-DNA it inhibits the RNA synthesis, probably by blocking the movement of RNA polymerase along the DNA strand. The RNA synthesis is suppressed at concentrations considerably below those that inhibit DNA replication (Reich and Goldberg, 1964).

4.2. Methods

4.2.1. Cultivation of the organism

Cultivation under normal conditions of growth has been described in section 2.1. A 1-litre culture flask is used, which is equipped with openings for a pH electrode (Radiometer GK 2302 C), for gas supply (N_2 - CO_2, 9 : 1) and for the supply of titrant (1 M NaOH) to maintain the pH value of the culture. The electrode is connected to a titrator (Radiometer TTT 1a) for automatic titration of the acidic products of metabolism excreted by the organism. The concentration of potassium phosphate of the medium is lowered to 0.25 g per l for incorporation of ^{32}Pi into the lipids as

described in section 2.2. For normal growth conditions inocula of 1% (by vol.) of a 16 h culture are used, while 10% inocula are added when growth is inhibited by lack of human milk or by unfavourable pH (8.0). The effect of antibiotics is studied on cells grown to the late log phase. After 10.5 h of cultivation under normal growth condition cells are sedimented by centrifugation at 13.000 g for 15 min, resuspended to the same optical density in fresh medium containing the antibiotic and cultivated again.

4.2.2. Isolation and fractionation of the phosphate-containing lipids

The membrane lipids are extracted as described in section 2.3. Washing of the cells with 0 2 M acetate buffer (pH 5.0) before extraction is performed only when lipid-hexose is assayed in the extract. Thin-layer chromatography is applied to the total lipid fraction without previous fractionation by column chromatography (section 2.5). The chromatograms are developed as given in Fig. 7. After development in the first direction the plate is reactivated for 2 min with hot air, while the part above the origin is covered with a glass strip to prevent degradation of the lipids. The ^{32}P-labeled spots are located by autoradiography, scraped off, and counted as indicated in section 2.4. For determination of the specific activity of the individual lipids separation is carried out by one-dimensional chromatography, The radioactive spots are scraped off, piled in small columns in chloroform-methanol (1 · 1, by vol.) and the lipids are eluted from the silicagel with chloroform-methanol-water (10 : 10 : 1, by vol.).

4.2.3. Analytical procedures

Lipid-phosphorus and lipid-hexose are determined in samples of 100-250 µmoles and 300-600 µmoles, respectively (see section 3.2.2). The molar amounts of the individual phosphate-containing lipids after 3.5 h of ^{32}P-incorporation are calculated from the extracted amount of radioactivity of each lipid devided by its specific radioactivity. Samples for radioactivity counting are taken from the cultures for an exact determination of the added amount of $^{32}P_i$. All values of incorporated radioactivity are normalized for 1 µCi $^{32}P_i$ added per ml culture. The total $^{32}P_i$ uptake into the cells during cultivation is calculated from the decrease of the ^{32}P-radioactivity of the medium (13.000 g supernatant). Radioactivity of the total lipids or of the individual lipids are counted as described in section 2.4.

4.3. Results

4.3.1. General characteristics

In a culture, inoculated at 37°C, the onset to rapid growth appears after a lag time about 4 h. The stationary phase is reached after approximately 14 h (Fig. 12). The optical density in the stationary phase usually varies between a 550 nm absorbance of 2.5 and 3.0. A 550 nm optical density of 2,50, measured in colorimeter tubes of 13 mm diameter, corresponds to $7.5.10^8$ bacteria per ml culture, counted as colonies on agar plates. Growth occurs at an exponential rate between 4 and 7 h after inoculation. The exponential growth, termed "log phase", shades off into linear growth, which is designated as "late log phase". The increase of wet weight of cells and of the extractable amount of total lipids during the growth cycle are given in Fig. 12 b and c.

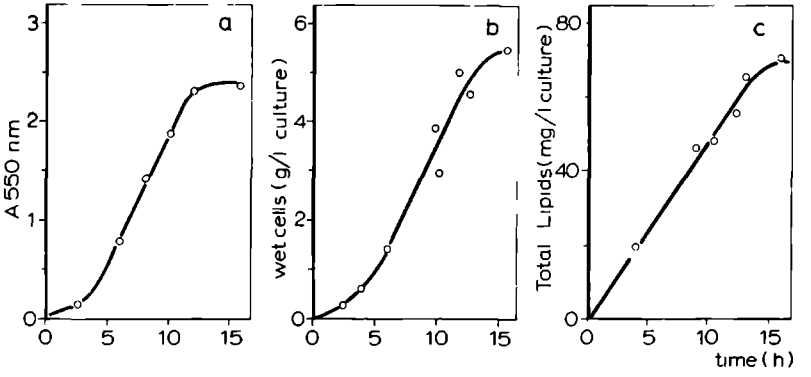

Figure 12.

Increase of cell mass and of lipids during growth cycle.

a) Optical density of the culture, b) Wet weight of cells after sedimentation at 13.000 g, c) Dry weight of total extracted lipids.

TABLE X.

Characteristics of a 16 h culture.

The relative amounts of radioactivity are given for a culture which is supplied with $^{32}P_i$ at the time of inoculation. The values are given as means for 3 cultures

Lipid-phosphorus (μ moles/l culture)	25 ± 3	
Lipid-hexose (")	45 ± 5	
Relative amounts of ^{32}P-radioactivity (%)		
uptake into the cells	50	
cell extract	8	
methanol/water layer		7
chloroform layer		1
cell residue	42	
	100	

The yields of bacteria and lipids from a culture in the stationary phase are given in Table X. Extractable lipid-^{32}P amounts to 1% of the added radioactivity. The methanol-water layer (7%) will contain intracellular inorganic phosphate and low molecular compounds (ATP, sugar phosphate). The bulk of the ^{32}P-uptake is recovered in the cell residue (DNA, RNA).

The phospho(galacto)lipid composition corresponds to the ^{32}P-distribution, when the lipids are homogenously labeled. Homogenous labeling requires more time, when growth is inhibited by lack of human milk or by cultivation at pH 8.0 (Fig. 13). Distribution of lipid-^{32}P and lipid-P is quite similar after 3.5 h under normal growth condition (pH 6.8 with human milk), but differs in the other cultures. Under optimal growth condition the lipids are labeled closest to isotope equilibrium. The height of ^{32}P-incorporation into the individual lipids will be considered later (section 5.3.2).

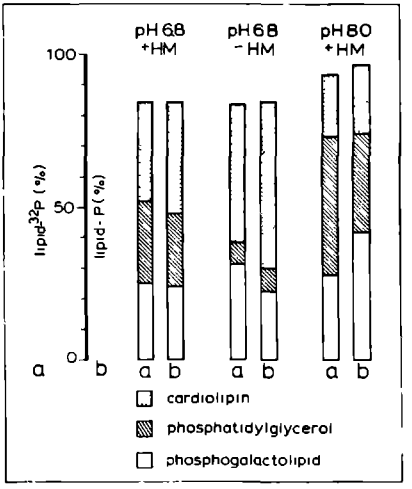

Figure 13.

Relative amounts of lipid-^{32}P and lipid-phosphorus after 3.5 h of ^{32}P-incorporation. 50 ml portions of a 16 h culture are diluted 5 times with fresh medium with and without human milk (HM). The pH is adjusted with NaOH to pH 6.8 and 8.0 as indicated. After addition of $^{32}P_i$ (1 μ Ci/ml) the cells are cultivated again for 3.5 h. Growth continued in this period for 1.0, 0.6 and 0.2 bacterial doubling in the media pH 6.8 + HM, pH 6.8 - HM, and pH 8.0 + HM, respectively, when the excreted acidic products of metabolism are not titrated during cultivation. The amount of lipid-phosphorus of each lipid is calculated from its amount of radioactivity divided by its specific activity.

The course of ^{32}Pi incorporation into the lipid fraction parallels that of the optical density curve (Fig. 14). The maximum value amounts approximately to 10.000 cpm/ml culture/unit of optical density when 1 µCi ^{32}Pi per ml culture is added. The decline during the stationary phase reflects an inefficient extraction (see Fig. 6). Therefore, a constant level of lipid ^{32}P-radioactivity has to be considered as a prerequisite for efficient extraction, particularly for the extraction of cardiolipin (CL) and lyso-cardiolipin (lyso-CL). The lag in overall growth (4 h) is somewhat larger than the lag in phospholipid synthesis (3 h), as shown by the lipid radioactivity curve. This is consistent with the finding of others (Schaechter, 1961; Okuyama; 1969) that the synthesis of cellular components preceeds overall growth.

During cultivation the pH of the medium drops as a result of excretion of acidic products of metabolism. Acetic acid is excreted as the main end-product of metabolism (Veerkamp, 1969; Poupard et al; 1973), which is buffered predominantly by the sodium acetate present in the medium.

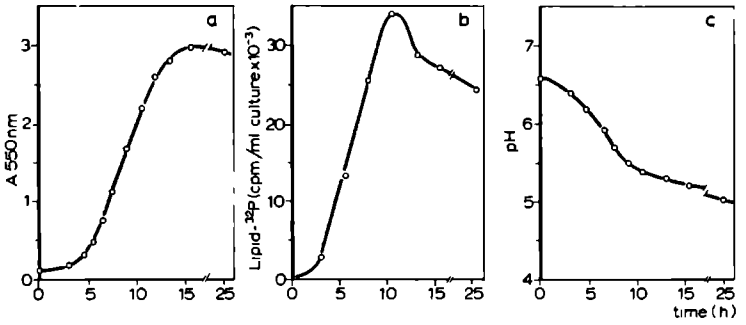

Figure 14.

Increase of incorporated radioactivity into the phospholipids during the growth cycle. a) Optical density of the culture, b) Incorporated radioactivity of total lipids, c) Medium pH during growth.

4.3.2. Phospholipid composition in relation to the growth phase

During exponential growth CL accounts for 46% of the total amount of lipid phosphorus. When the cells are ageing, the relative amounts of CL remain rather constant while lyso-CL increases (Table XI). Phosphatidylglycerol (PG) becomes a minor component in the stationary phase. Glycerophosphorylgalactosyldiglyceride (compound 15) decreases somewhat, when the cells grow older. This is accompanied by an increase in its lyso-derivative.

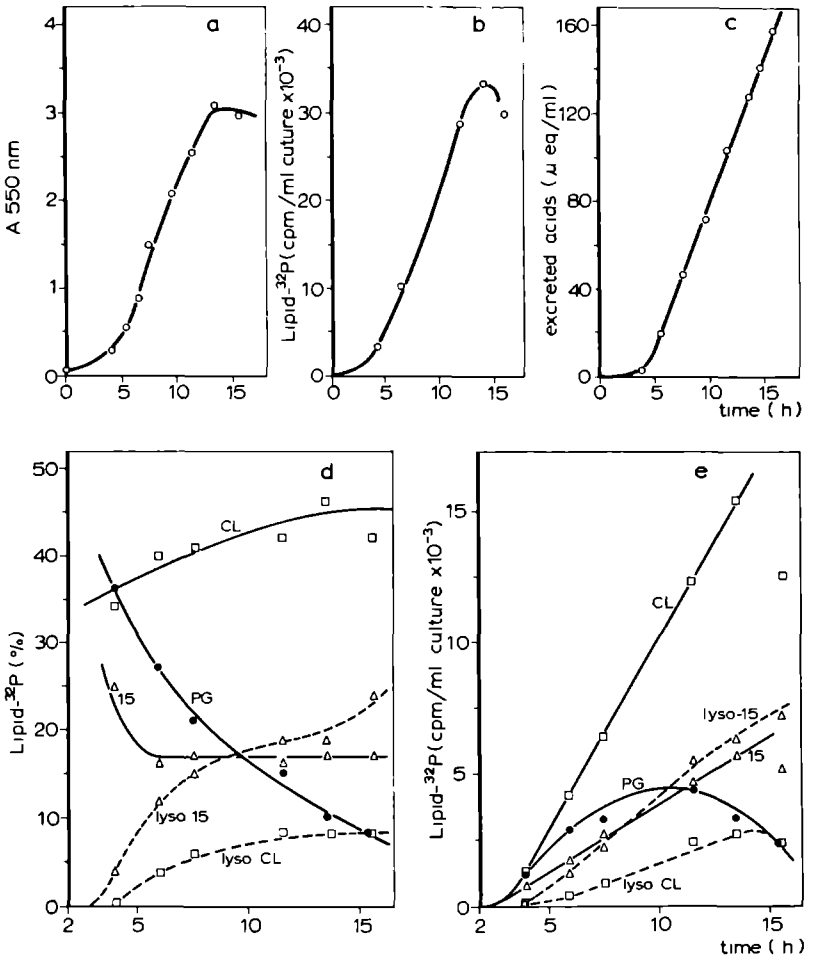

Figure 15.

Culture at constant pH of 6.8 a) Optical density of the culture, b) and e) Amounts of ^{32}P-radioactivity incorporated into the total lipids and into the individual lipids, respectively, c) Amount of excreted acidic products of metabolism automatically titrated with 1 M NaOH, d) Relative amounts of lipid-^{32}P in the individual phospho(galacto)lipids

TABLE XI.

Change of phospholipid composition of the cells during growth from the log phase to the stationary phase.

The phospholipids are given as relative amounts of radioactivity incorporated into the lipid fraction. Data are obtained from Fig. 6d.

	Log phase	Onset to stationary phase	Stationary phase
pH of culture medium	pH 6.1	pH 5.2	pH 4.9
Cardiolipin	46	44	43
Lyso-cardiolipins	5	10	20
Phosphatidylglycerol	21	9	4
Glycerophosphorylgalactosyl diglyceride	28	21	17
Glycerophosphorylgalactosyl monoglyceride	0	16	16
	100	100	100

In order to investigate the effect of decreasing pH upon the change in phospholipid composition we have kept the pH value of the culture constant by automatic titration of the excreted acids. At a constant pH of 6.8 acids are excreted at a constant rate, which does not level off at the onset of the stationary phase (Fig. 15 c). The curves for the optical density and for the ^{32}P-incorporation into the phospholipids (Fig. 15 a and b) are comparable with those found for cultures without neutralization of the excreted acids (Fig. 6 a and b and Fig. 14 a and b). Considering the changes in the relative amounts of lipid-radioactivity (Fig. 15 d), PG decreases from 40% in the lag phase to less than 10% in the stationary phase. The sum of CL and lyso-CL increases during ageing from 35 to 55%. Compound 15 decreases from approximately 25% during the lag phase to 18% during the log phase and then remains rather constant. Its lyso-derivative, increasing steadily during growth, becomes predominant over compound 15 during the stationary phase. The first sample is withdrawn from the culture after 4 h, so we may assume that the relative amounts of lipid-^{32}P agree with those of lipid-phosphorus (see Fig. 13). The changes in the phospholipid composition in this culture at constant pH 6.8 indicates that the effect of decreasing pH on the changes in phospholipid composition during growth as a result of carbohydrate fermentation is small.

The rates of ^{32}P-incorporation into the individual phospholipids are quite constant at pH 6.8 up to the stationary phase, except for PG (Fig. 15e). After a period of net increase of PG, its breakdown or conversion to other lipids starts to prevail. Therefore, PG metabolism dictates the changes in the relative amounts of the other phospholipids during ageing.

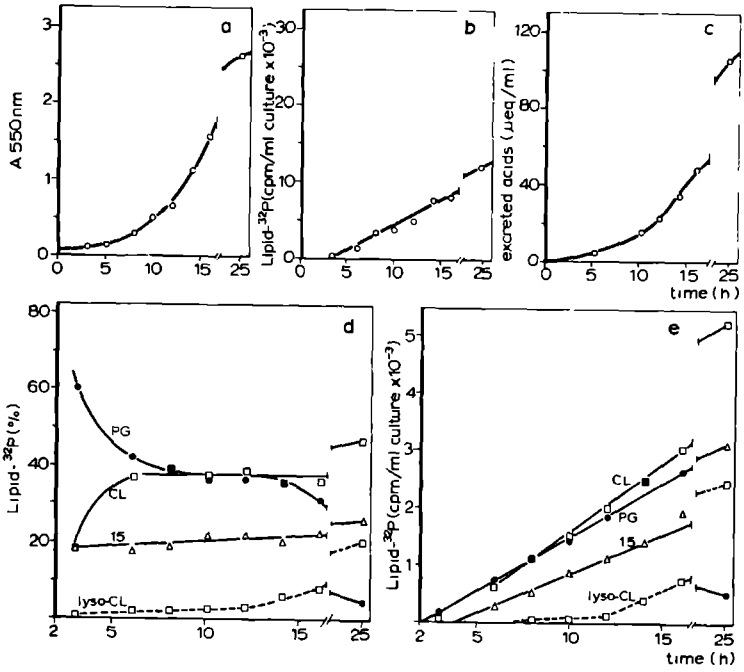

Figure 16.

Culture at constant pH of 5.5. Legends see Fig. 15.

TABLE XII.

Doubling times of *B.bifidum* var.*pennsylvanicus* during exponential growth.

Data are obtained from Figures 15, 16 and 17.

Growth condition	Doubling time
37°, constant pH 6.8	80 min
37°, constant pH 5.5	200 min
29°, non-constant pH	450 min

For a comparative study of pH effects, the organism is also cultivated at a constant pH of 5.5. After adaptation of its metabolism to the acidic environment during the first 5 hours, the organism starts to grow exponentially, though with a 2.5-fold longer doubling time than at pH 6.8 (Table XII). The duration of the log phase is increased to 16 h after inoculation (Fig. 16). The acid production during growth to the stationary phase corresponds with the amount observed at pH 6.8, but the total amount of incorporated radioactivity in the stationary phase per unit of optical density (5000 cpm/ml/unit O.D) is half of the value after cultivation at pH 6.8.

The ^{32}P-incorporation into the individual phospholipids at pH 5.5 occurs at a linear rate at least in the log phase (Fig.16 a and e). The incorporation in CL, lyso-CL and 15, further increases during the late log phase,while a decrease of the amount of PG occurs just as at pH 6.8. Remarkable, the lyso-phosphogalactolipid appears only in very small amounts, when a constant pH of 5.5 is maintained from the start of cultivation (less than 3% in the stationary phase). The relative amount of PG is equal to that of CL during the log phase. The discrepancy in the relative amounts of both lipids in the lag phase has to be ascribed mainly to a significant difference in turnover as will be shown in chapter 5. PG is most rapidly and CL is most slowly labeled with ^{32}Pi. This means that the difference in relative amounts of lipid-phosphorus of CL and PG is less than is suggested by the incorporated radioactivity.

TABLE XIII.

Effect of pH on ^{32}Pi incorporation into the phospholipids when cells are grown to the stationary phase.

The amounts of radioactivity are given in cpm x 10^{-3}/ml culture/unit of optical density. One nmole lipid-phosphorus corresponds to 1400 cpm. Data are obtained from Figures 6, 15 and 16, at those times when the optical densities amount to 3.20, 3.05 and 2.65, respectively.

	Final pH of 4.9	Constant pH of 6.8	Constant pH of 5.5
Cardiolipin	4.3	5.0	2.1
Lyso-cardiolipins	2.1	0.9	0.9
Phosphatidyl glycerol	0.4	1.1	0.2
Glycerophosphorylgalactosyl-diglyceride	1.7	1.8	1.2
Glycerophosphorylgalactosyl-monoglyceride	1.5	2.1	0.1
Total lipid-^{32}P	10.0	10.9	4.5

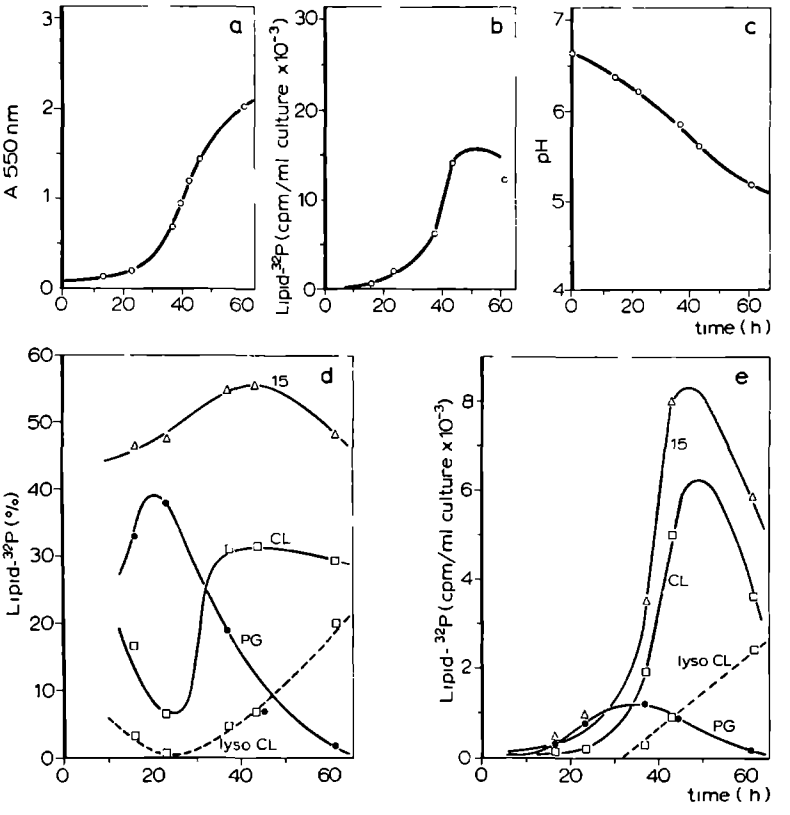

Figure 17.

Culture at 29°C at non-constant pH Legends see Fig. 15.

A summary of the results of ^{32}P-incorporation at non-constant pH, at pH 6.8 and at pH 5.5 are given in Table XIII for cells grown to the stationary phase. Small differences are found between the amounts of radioactivity incorporated into the total lipid fraction and into the individual phospholipids for the cultures at non-constant pH and at pH 6.8. However, remarkable differences are shown in the culture at pH 5.5 compared to the other two cultures. Apparently, these differences in the stationary phase must be ascribed mainly to the adaptation to the acidic environment during lag time.

A long lag time of about 15 h is observed, when the organism is cultivated at 29 °C (Fig. 17a). Subsequently, the culture grows exponentially for 20 h with a doubling time of 450 min (Table XII). The decrease of the pH during the growth cycle (Fig. 17c) is similar to that during cultivation at 37 °C (Fig. 14c). During cultivation at 29 °C the relative amount of the phosphogalactolipid (compound-15) is much higher than at 37 °C, accounting for 45-55% of lipid-^{32}P (Fig. 17d). Lyso-compound-15 is present at this temperature only in very small amounts (less than 3%). During lag time the initial increase in the relative amount of PG is counterbalanced by a decrease of CL. The decrease of the amounts of ^{32}P-activity of CL and 15 at the onset to the stationary phase may be ascribed to inefficient extraction as is suggested by the course of total lipid radioactivity.

4.3.3. Phospholipid composition under growth inhibitory conditions.

Cultivation of *B.bifidum* var.*pennsylvanicus* in the absence of human milk or sodium acetate prevents exponential growth of the organism. Inhibition of growth by lack of human milk is a result of starvation for certain glucosamine-containing cell wall precursors (section 1.1.). When the medium devoid of human milk is inoculated with a 16 h culture the optical density increases about twice (Figs 18a and 19a) owing to the residual cell wall present in the inoculum. The cell wall precursors are exhausted after approximately one bacterial doubling, after which growth is limited by lack of growth factors just as in the stationary phase under normal conditions of growth.
In the absence of sodium acetate growth is inhibited by a rapid fall of pH, since the excreted acetic acid produced by fermentation is no more buffered by sodium acetate.

The effect of pH on the phospholipid composition under these conditions inhibitory to growth is investigated as described above (section 4.3.2). The incorporated radioactivity of total lipids and of the individual phospholipids per cell unit is presented in Table XIV. The incorporated radioactivity is proportional to the amounts of lipid phosphorus, assuming that 10 hours are sufficient for a homogenous labeling of the phospholipids.
A comparison with Table XIII shows that the cellular amount of lipid phosphorus is increased under all inhibitory conditions studied. Since all cultures are inoculated with cells grown to the stationary phase the initial lipid-phosphorus content corresponds to 10.000 cpm/ml/ unit optical density (Table XIII). The increase of the cellular amount under the growth inhibitory conditions varies from a factor 1.4 to a factor 1.8 (Table XIV), and is mainly a result of the increased amount of CL and PG per cell. In the absence of human milk the effect of pH upon the individual phospholipids resembles that found in the cultures supplied with human milk (Tables XII and XIV). Small differences are found between the amounts of radioactivity incorporated into the total lipid fraction and into the individual phospholipids for the cultures at non-constant pH and at pH 6.8 (Table XIV). However, cultivation at a constant pH of 5.5 from the start results in a significant decrease of the cellular amount of PG and particularly of lyso-compound 15.

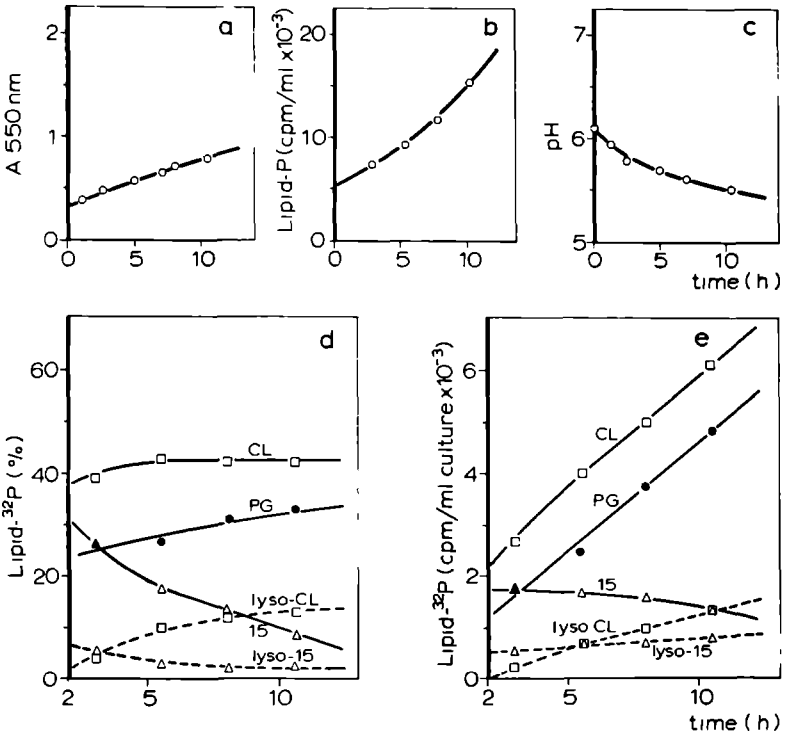

Figure 18.

Culture in medium devoid of human milk at non-constant pH. A 16 h culture is used as inoculum. Legends see Figures 14 and 15.

Cultivation of the organism at a constant pH of 8.0 in the presence of human milk has the same inhibitory effect on overall-growth as withholding human milk during cultivation at pH 5.5. (Figs 19a and 20a).
The ^{32}P-incorporation into the total lipid fraction differs somewhat in both cultures (Figs 19b and 20b), as does the acid production during cultivation (Figs 19c and 20c). The most striking difference between both growth conditions finds its expression in the ^{32}P-phospholipid composition (Figs 19d and 20d) and in the rates of ^{32}P-incorporation into the individual phospholipids (Figs 19e and 20e). At pH 8.0 in the presence of human milk a linear decrease of the relative amount of PG from 70 to 40% of total lipid radioactivity in 10 h is accompanied by a linear increase of CL from 10 to 40%. The relative amount of compound 15 remains rather constant (18-15%), while lyso-compound 15 represents less than 3%. Following the ^{32}P-incorporation in each phospholipid we found an increase of ^{32}P-radioactivity in CL and lyso-CL, but a decrease in PG. The change in ^{32}P-radioactivity of compound 15 is small (Fig. 20e). The high amount of ^{32}P-activity in PG after 3 h of incorporation, which is characteristic for the alkaline condition, will be due to a high turnover of PG. The rate of ^{32}Pi-incorporation into CL increases from about 200 cpm/ml/hour after 3 h to about 400 cpm/ml/hour after 7 h, which indicates significant net synthesis of CL.

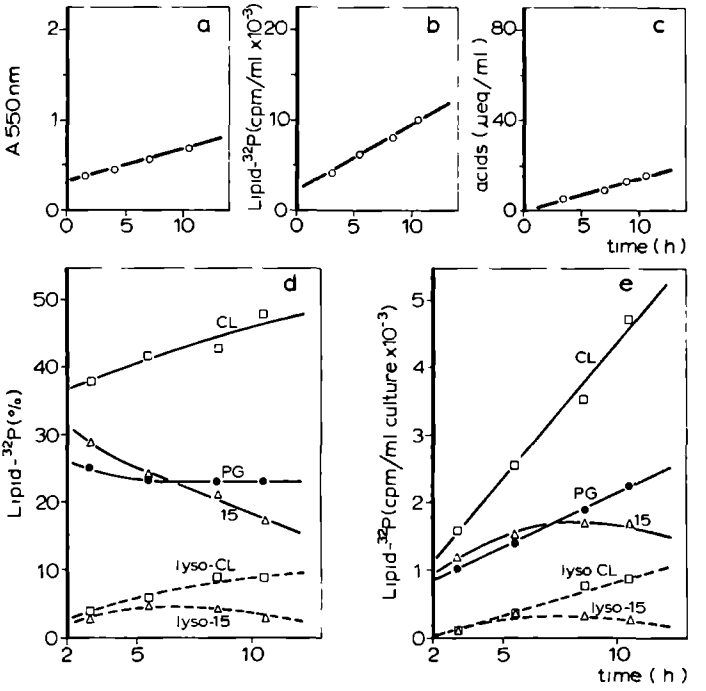

Figure 19.

Culture at constant pH of 5.5 in medium devoid of human milk. A 16 h culture is used as inoculum. Legends see Fig. 15.

TABLE XIV.

Effect of pH on $^{32}P_i$-incorporation into the phospholipids under growth-inhibitory conditions.

Media devoid of human milk (-HM) and without sodium acetate (-AC) are inoculated with a 16 h culture, 10% and 1%, by vol. respectively. The organism is cultivated with $^{32}P_i$ for 16 h in the medium without sodium acetate and for 10.5 h in the medium devoid of human milk. The optical density lies between 0.72 and 0.80 at time of harvest. The incorporated radioactivity is given in cpm x 10^{-3}/ml culture/unit of optical density. Data of the culture devoid of human milk at non-constant pH and at pH 5.5 are obtained from Figures 18 and 19.

	-AC Final pH 4.3	-HM Final pH 5.5	-HM Constant pH 6.8	-HM Constant pH 5.5
Cardiolipin	5.9	7.8	7.9	6.5
Lyso-cardiolipins	1.6	1.7	0.8	1.2
Phosphatidylglycerol	5.2	6.1	5.0	3.2
Glycerophosphorylgalactosyl diglyceride	2.2	1.7	3.2	2.4
Glycerophosphorylgalactosyl monoglyceride	1.1	1.1	1.0	0.4
Total lipid-^{32}P	16 0	18.4	17 9	13.7

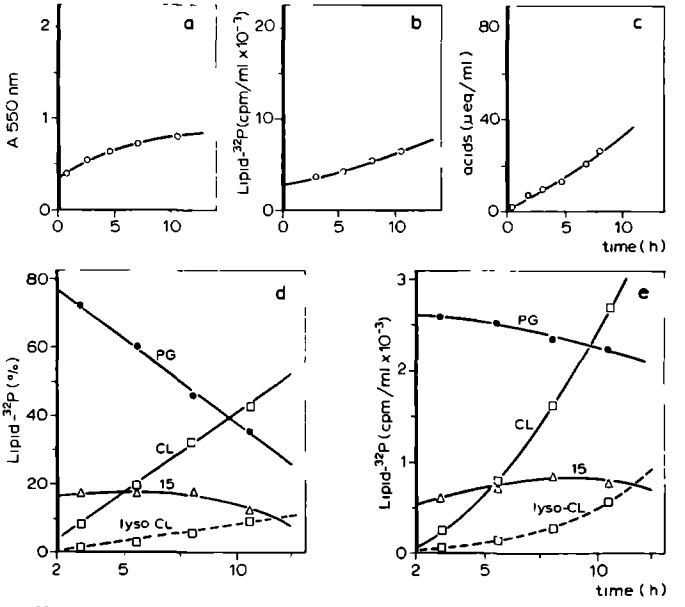

Figure 20.

Culture at constant pH of 8.0 in the presence of human milk. A 16 h culture is used as inoculum. Legends see Fig. 15.

When both growth-inhibitory conditions, pH 8.0 and lack of human milk, are present simultaneously, no increase in optical density is observed during cultivation (Fig. 21a). The ^{32}P-incorporation into the total lipid fraction is low (Fig. 21b). The excretion of acids into the medium is still significant, indicating that cell metabolism is not blocked, i.e. the cells are still viable.

PG constitutes even 80% of phospholipid radioactivity. The decrease of the relative amount of PG during cultivation is slower than observed in the culture at pH 8.0 in the presence of human milk (Fig. 20d), but is also counterbalanced by the increase of the relative amount of CL. The radioactivity is incorporated into the individual phospholipids at comparable rates between 3 and 10.5 h after ^{32}Pi administration. The high incorporation into PG between 0 and 3 h will be due to high turnover. The complete inhibition of overall growth is accompanied by a very low incorporation of ^{32}Pi into CL. In the culture at pH 8.0 in the presence of human milk CL synthesis reflects membrane synthesis inherent to the small overall growth (Fig. 20c). The requirement for CL synthesis is less when overall growth is fully inhibited at pH 8.0 in the absence of human milk (Fig. 21c).

After 3.5 h of ^{32}P-incorporation at pH 8.0 there exist still a discrepancy between the relative amounts of lipid-^{32}P and lipid-phosphorus in the main phospholipids (see Fig. 13), which is the result of different turnover rates of the individual phospholipids. After continued incorporation the differences of the relative amounts of lipid ^{32}P and lipid-phosphorus

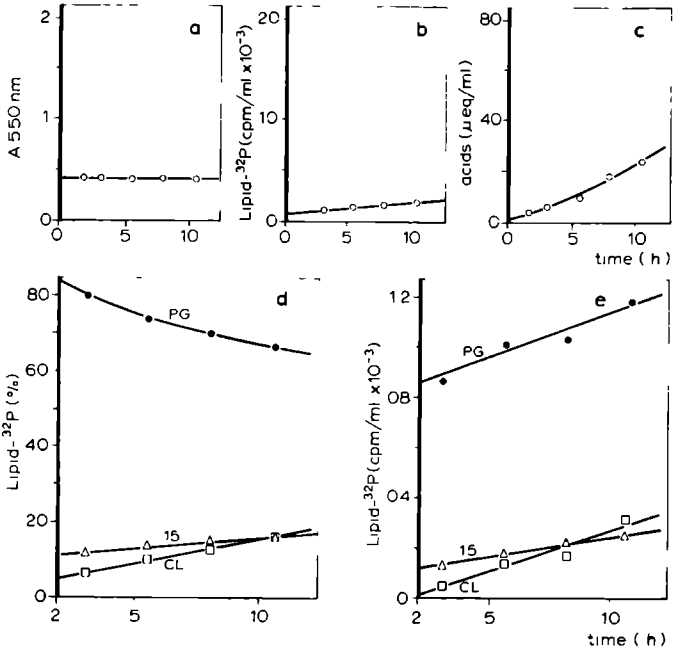

Figure 21.

Culture at constant pH of 8.0 in medium devoid of human milk. A 16 h culture is used as inoculum. Legends see Fig. 15.

diminish, so that the rate of ^{32}P-incorporation approaches more and more the rate of net synthesis or net breakdown.

The effect of pH 8.0 is also investigated after approximately homogenous labeling. Therefore, the organism is cultivated for 9 hours at a constant pH of 6.8 in the presence of human milk before shifting the pH from 6.8 to 8.0 by addition of NaOH when the optical density at 550 nm is 1.0. The growth slows down immediately by the alkaline condition, but the rate of acid production does not change (Figs 22a and c). The incorporated radioactivity in the total phospholipid fraction is given in Fig. 22b. The instantaneous decrease after the pH shift must be caused by a decreased extractability of the lipids after shifting of the pH. Following the radioactivity of the individual phospholipids, the increase of radioactivity in PG after the pH shift is striking, even more so as the total lipid radioactivity is diminished. This points to an instantaneous conversion of CL to PG caused by shifting the pH from 6.8 to 8.0. Since the phospholipids are approximately homogeneously labeled after 9 h, when three duplications have taken place, the increased amount of radioactivity of PG cannot be the result of an increased turnover rate due to the pH shift.

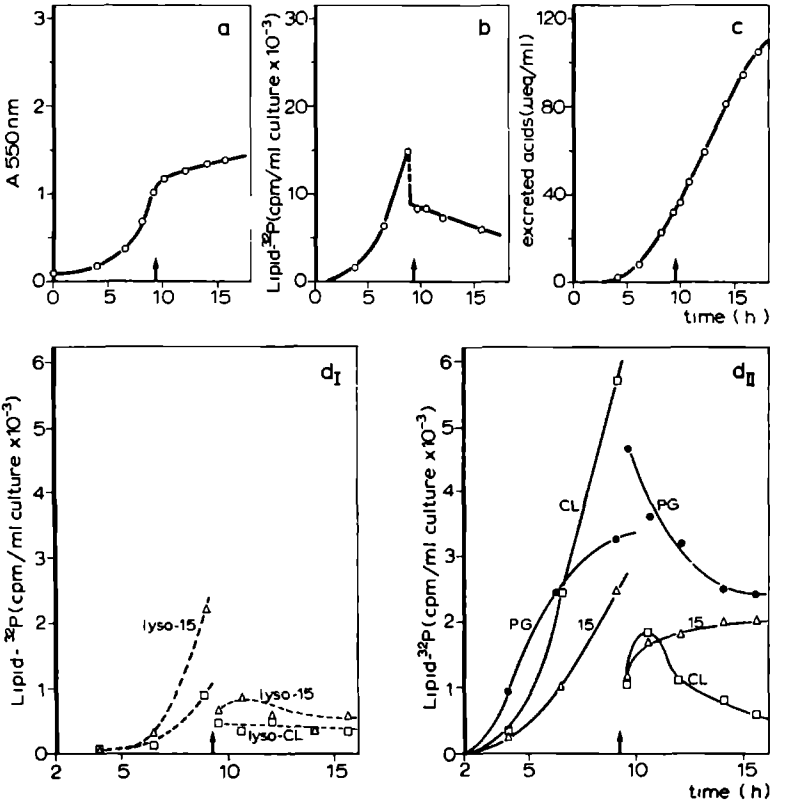

Figure 22.

The effect of shifting the pH from 6.8 to 8.0 in a rapidly growing culture. The shift is achieved by addition of NaOH after 9 h of cultivation, indicated by the arrow. Legends see Figure 15.

4.3.4. The effect of antibiotics on phospholipid composition.

The effect of antibiotics on the amount of total lipids and individual lipids per cell, is studied by growing the cells for 10.5 h in a 800 ml culture and subsequently harvesting them in portions of 75 ml. The harvested cells are resuspended in the same volume (75 ml) of fresh medium containing the antibiotic under investigation (Table XV) and cultivated again for 3.5 h. One portion is resuspended in medium without human milk and antibiotic. Before and after resuspension the cells are cultivated in the presence of ^{32}Pi of equal specific activity.

In the presence of penicillin and vancomycin no increase in optical density is observed at all. The radioactivity of the extracellular milieu increases by 10% and 4% (Table XV), indicating a net efflux of phosphate from the cells in the presence of these antibiotics. The increase of the extracellular amounts of radioactivity in the presence of penicillin and vancomycin corresponds to 160 and 60 nmoles phosphate/ml culture, respectively, assumed that the specific activity of the excreted phosphate is equal to that of the incorporated ^{32}Pi (1400 cpm/nmole phosphate). The pool size of the total intracellular phosphate can be estimated from the control cultures (Table XV). An increase of the optical density of the control culture of 1.85 to 3.90 at 550 nm is accompanied by an uptake of 40% of the extracellular

TABLE XV.

Effect of antibiotics on phosphate uptake and on lipid-phosphorus and lipid-hexose contents of the cells.

Cells grown to the late log phase in the presence of $^{32}P_i$ are resuspended in fresh medium with the same specific activity of $^{32}P_i$ and cultivated subsequently for 3.5 h in the presence of an antibiotic or in the absence of human milk. During cultivation the acidic products of metabolism are not neutralized, resulting in a final pH between 5.4 and 5.7 after 3.5 hours. The optical density at 550 nm of the control culture is increased from 1.85 to 3.90 in 3 5 h. Phosphate uptake into the cells is calculated as difference of ^{32}P-radioactivity in the supernatant (13.000 g) at times 0 h and 3.5 h. Lipid-phosphorus and lipid-hexose contents after 3.5 h are given in μ moles/l culture/unit of optical density.

Antibiotic added	*Conc.* (μg/ml)	*Growth* (doublings)	*Pi-uptake* (%)	*Lipid-phosphorus*	*Lipid-hexose*
Control (0 h)	-	-	-	6.5	20
Control (3.5 h)	-	1 09	40	6.1	25
Without human milk	-	0.45	18	15.0	18
Penicilli n	2*)	0.0	-10	10.2	25
Cycloserine	80	0 30	15	10.6	24
Vancomycin	20	0 0	- 4	8.2	16
Bacitracin	400*)	0 41	15	7.8	19
Chloramphenicol	300	0.75	56	8.0	22
Streptomycin	200	0.64	35	5.8	27
Actinomycin-D	10	1 05	45	5.7	20

*) Penicillin and bacitracin preparations contain 500.000 U and 20.000 U per gram, respectively.

TABLE XVI.

Effect of antibiotics and lack of human milk on the phospho(galacto)lipid content of the cells.

Data are obtained from the experiment given in Table XV. The cellular amounts of the phospholipids are given in cpm x 10^{-3} per ml culture and per unit of optical density.

Antibiotic added	CL	Lyso-CL	PG	Comp.15	Lyso Comp.15	Total
Control (0 h)	1.3	0 2	2.3	1 5	1.0	6.3
Control (3.5 h)	0.8	0 2	3.1	3 3	1.6	9.0
Without human milk	9.2	1 7	2.6	2.7	1.7	17.9
Penicillin	7.4	3 0	2.6	1.5	0.4	14.9
Cycloserine	6.4	1.6	3.7	2.5	0.7	14.9
Vancomycin	4.7	1 0	1.6	1.7	0.3	9.3
Bacitracin	1.2	0 3	2.2	3.4	1.3	8.4
Chloramphenicol	2.2	0 3	3.0	3 1	0.9	9.5
Streptomycin	0.3	0 0	2.3	2.9	1.2	6.7
Actinomycin-D	1.2	0 3	2.4	3.3	1.0	8.2

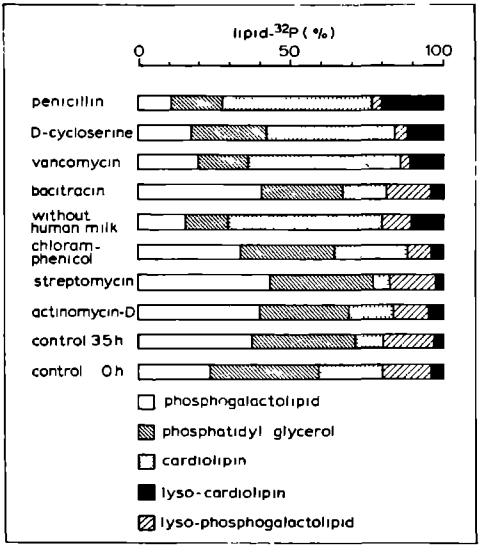

Figure 23.

Effect of antibiotics and lack of human milk on the phospho(galacto)lipid composition. Data are obtained from Table XVI.

^{32}Pi, which corresponds to 630 nmole phosphate/ml culture. This will also be approximately the initial, total cellular phosphate pool (control oh), suggested by the two-fold increase of the optical density. This means that the amounts of phosphate excreted from the cells in the presence of penicillin and vancomycin in 3.5 h are equivalent to about 25 and 10% of the initial total cellular phosphate pools.

In the presence of the cell wall inhibitors cycloserine and bacitracin growth continues to some extent, and is accompanied by 15% net uptake of medium phcsphate into the cell. Moderate growth inhibition is observed in the presence of the protein synthesis inhibitors chloramphenicol and streptomycin, while growth is hardly inhibited in the presence of actinomycin-D (Table XV).

The content of lipid-phosphorus per cell is increased when cell wall synthesis is inhibited by antibiotics, particularly by penicillin and cycloserine and also by lack of human milk, as has been reported earlier (Table XIV). The antibiotics have less effect on the lipid-hexose content of the cells than on the lipid-phosphorus content (Table XV).

The amounts of radioactivity incorporated into the individual phospholipids are shown in Table XVI. These values will be proportional to the chemical amounts of lipid-phosphorus, since ^{32}P-incorporation has taken place during 10.5 h prior to the addition of antibiotics. This table shows that the increase of lipid-^{32}P per cell after cell wall inhibition is the result of a 4 to 7-fold increase of the CL + lyso CL content per cell. Bacitracin forms an exception in this respect, since the cellular amounts of the individual phospholipids are equal to those in the control culture after 3.5 h. The relative amounts of the phosphogalactolipids (compound 15 + lyso-compound 15) are high in the presence of bacitracin, chloramphenicol, streptomycin, and actinomycin D (Fig. 23), but the amounts per cell are equal to those of control culture after 3.5 h (Table XVI).

4.4. Discussion and conclusions.

The phosphogalactolipid (compound 15), the structure of which is given in chapter 3, occurs in *B.bifidum* var.*pennsylvanicus* at all cultural ages and constitutes 18-25% of the phosphorous-containing lipids in the log phase and stationary phase. Its lyso-derivative increases gradually to 25% of lipid-phosphate in the stationary phase. The relative amounts of both phosphogalactolipids as well as the total amounts per cell are scarcely different, whether the initial pH (6.8) is maintained during growth or not. At a constant pH of 5.5 the same relative amount (29%) of the phosphogalactolipid is found, but the lyso-derivative does not appear. At 29 °C the lyso-derivative is also absent, but the phosphogalactolipid itself accounts for 50-55% of the lipid-phosphate during log and late log phase. So far comparably high amounts of phosphoglycolipids have been found only in *mycoplasmas* (Smith et al, 1973). In *A.laidlawii* glycerophosphoryl diglucosyl diglyceride comprises 50% of the phosphorous containing lipids. *Thermoplasma acidophilum* contains a phosphoglycolipid that accounts for even 90% of the lipid-phosphate. Glycerophosphoryl diglucosyl diglyceride has been isolated as a minor compound (1-3%) from three strains of *Streptococcus* (Fischer et al, 1973a). The core structure of this lipid is given in Fig. 8). Glucosyl-phosphatidylglycerol accounts for 14% of lipid phosphate in an unidentified halophilic bacterium (Peleg and Tietz, 1973), while the relative amount of phosphatidylglucosyldiglyceride isolated from *Ps.diminuta* amounts to 10% of the lipid phosphate (Wilkinson and Bell, 1971). However, the classification of this latter organism seems to be questionable (Smith et al, 1973).

The changes in the phospho(galacto)lipid pattern in batch cultures are primarily determined by the growth phase rather than by the change of pH during cultivation. PG is the only phosphate-containing lipid, the net synthesis of which does not occur at a constant rate during rapid growth. A net increase in PG in the exponential phase is followed by a decrease in the late log phase. A conversion of PG to CL, required for membrane synthesis, may occur more rapidly than the *de novo* synthesis of PG. A linear increase in ^{32}P-incorporation into PG is found at pH 5.5, possibly as a result of the lower rate of growth. In this culture the formation of lyso-phosphogalactolipid is blocked. Furthermore, the cellular lipid-phosphorus content in the stationary phase at pH 5.5 is only half that found after cultivation at non-constant pH or at pH 6.8.

Changes in the lipid-phosphorus distribution may reflect variations in cell metabolism or membrane function. For instance, *B.stearothermophilus* has two stages of exponential growth. In the first stage glucose is fermented to acetic acid, which is further oxidized in the second stage (Lee and Oo, 1973). This change in metabolism is accompanied by a marked increase in PG.

In *S.aureus*, the formation of a functional membrane-bound electron transport system takes place after a shift from anaerobic to aerobic growth. A simultaneous increase in the amounts of mono- and diglucosyldiglyceride, CL, PG and vitamin K_2 parallels the changes in the respiratory pigments (Frerman and White, 1967).

Damage to the nutrient transport systems in the membrane will also cause a change in cell metabolism, since induction of intracellular enzymes is conditioned by the uptake of their substrates. In *E.coli* β-galactosidase induction is preceded by the induction of galactoside-permease. In the induced cells there may be as many as 8000 permease sites on the surface of the cell, so that the total permease complement accounts for an appreciable fraction of the membrane protein (Fox et al, 1967).

The cytoplasmic membrane is relatively impermeable to hydrogen and hydroxyl ions, but unfavourable environmental pH values will damage not only transport systems but also enzymes involved in cell wall and membranelipid synthesis.

The effect of the environmental pH on the phospholipid content in *S.aureus* has been most carefully studied. Wide variations in the proportions of PG and lysyl-PG at different stages of growth have been ascribed to changes in the pH of the growth medium, whether these were due to glucose fermentation or to artificial means as carried out in our study (Houtsmuller and Van Deenen, 1965). Lysyl-PG predominates at low pH values and PG near neutrality. Gould and Lennarz (1970) showed that these changes in the proportion of PG and lysyl-PG are mainly the result of a large decrease in the absolute amount of PG per cell, concomitant with a small increase in lysyl-PG. The permeability of the intact cells for erythritol appears to increase with an increasing lysyl-PG to PG ratio (Haest et al, 1972).

In *B.bifidum* the transport of inorganic phosphate through the membrane may be dependent on K^+ and Na^+, as in *E.coli* (Weiden et al, 1967; Medveczky and Rosenberg, 1971). The stimulatory effect of K^+ and the inhibitory effect of Na^+ in this organism may imply the involvement of an Na^+-K^+ ATPase in phosphate uptake. In our experiments at pH 6.8 in the presence of human milk the excreted acids are titrated with NaOH, raising the Na^+ concentration from 0.31 to 0.47 M. The K^+ concentration, originating from K_2HPO_4, remains constant (2.9 mM). This change in the Na^+/K^+ ratio in the medium has no significant effect on the phospholipid synthesis and the phospholipid composition during growth. Increasing Na^+ concentration also changes the osmolarity of the medium. An increase in the relative amount of CL and a simultaneous

decrease in PG in *S.aureus* has been observed upon increasing the NaCl concentration from 1 to 2 M (Kanemasa et al, 1972).

A decrease in the relative amount of PG and a concomitant increase in the relative amount of CL is characteristic for several bacteria grown to the stationary phase (Cronan, 1968; Randle et al, 1969; Short and White, 1971; Card, 1973). However, a rapid loss of CL and an increase of PG during transition from the exponential to the stationary phase has been demonstrated in *B.licheniformis* (Mormon and White, 1970). The metabolic interrelationship between CL and PG has been extensively investigated in *S.aureus* and *H.parainfluenzae*. In these organisms PG and CL can be interconverted in a cyclic manner (Short and White, 1971, 1972; Ono and White, 1970, 1971). Cardiolipin is synthesized by combination of two molecules of PG with the release of glycerol. This mechanism of CL synthesis has also been demonstrated to occur in *E.coli* (Hostetler et al, 1972; Hirschberg and Kennedy, 1972; Tunaitis and Cronan, 1973) in addition to the PG plus CDP-diglyceride pathway (Fig. 3). In *M.lysodeikticus* (De Siervo and Salton, 1971) and in *B.stearothermophilus* (Card, 1973) the synthesis of CL from 2 moles of PG has also been demonstrated. A regeneration of PG and PA (phosphatidic acid) from CL is catalyzed by a CL-specific phospholipase D, which enzyme has been isolated from *H.parainfluenzae* (Ono and White, 1970; Astrachan, 1973). The asymmetric hydrolysis of CL has also been demonstrated in *E.coli* in vivo (Stanacev et al, 1967). A phenotypic variation of the proportions of PG and CL has been achieved in *H.parainfluenzae* by addition of EDTA to exponentially growing cells. EDTA binds magnesium and thus inhibits the CL-specific phospholipase D, which requires Mg^{2+} as cofactor, which results in an accumulation of CL (Ono and Whita, 1971; Tucker and White, 1971).

In *B.bifidum* var.*pennsylvanicus* the occurrence of a similar asymmetric hydrolysis of CL is suggested by shifting the pH from 6.8 to 8.0 (Fig. 22). However, PA is not detected although PG and PA are formed in equimolar amounts during hydrolysis of CL. This can be explained by a rapid reutilization for phospholipid synthesis (see Fig. 3).

During cultivation at a constant pH of 8.0 the relative amount of PG is high during the first hours (Figs 20d and 21d), but the relative amount of CL increases steadily and becomes predominant in the presence of human milk, which enables the organism to grow to some extent (Fig. 20a and d). This suggests that CL synthesis is a prerequisite for membrane synthesis.

Comparison of the phospholipid and phosphogalactolipid metabolism reveals that the rate of synthesis of CL is more than twice as high as that of compound 15 and its lyso-derivative during rapid growth at optimal pH. Lyso-compound 15 is not synthesized or only in small amounts under unfavourable conditions of growth (lack of human milk, pH 5.5, temperature of 29 °C, penicillin). The phospho(galacto)lipid composition differs greatly in cells grown at 29° and 37°, mainly by the high proportion of the phosphogalactolipid at the lower temperature. Its synthesis then even exceeds the rate of CL synthesis in the exponential and the late log phase. Cultivation at 29 °C probably affects the overall cell metabolism, but the effect may be different for the various enzyme activities in the cell. Changes in phospholipid composition have been reported for *E.coli* when grown at 27° and 37° (De Siervo, 1969), but not after lowering the temperature from 37° to 10° (Okuyama, 1969). Furthermore, small changes in the phospholipid composition have been observed in *S.aureus* after a shift from 37° to 25°C (Joyce et al, 1970), in *Ps.fluorescens* grown at various temperatures between 5° and 23° (Cullen et al, 1971), and in *B.stearethermophilus* grown at 37° and 55°C (Lee and Oo, 1973).

A metabolic relationship between PG and compound-15 is suggested by pulse-chase experiments (Chapter 5), PG being the phosphate donor of the phosphogalactolipid. The decrease of the total amount of PG during cultivation at 29 °C may reflect its conversion to both CL and compound-15. It can not be excluded that PG and compound-15 can be interconverted in a cyclic manner as is the case for PG and CL.

The total amount of the phosphogalactolipid decreases after a period of net synthesis, when growth is inhibited by lack of human milk or at pH 8.0 (Figs 19, 20, 21). This reflects either a transfer of the phosphate moiety to another lipid, possibly PG, or a conversion to a water-soluble product.

With regard to the effects of antibiotics on the phospho(galacto)lipids, an increase of the CL/PG ratio in the presence of penicillin, as is demonstrated in this study, has also been shown in *E.coli* (Starka and Moravova, 1970). In *B.bifidum* var.*pennsylvanicus* the amounts per cell of CL and lyso-CL are increased more than 5 times, while the overall growth is completely inhibited (Table XVI). The CL and lyso-CL contents are also increased by cycloserine and vancomycin, the other inhibitors of cell wall synthesis, though in a less pronounced way. Bacitracin demonstrates a different behaviour in this respect. The highest increase in the CL and lyso-CL content has been found, when cell wall synthesis is inhibited by lack of human milk (7-fold increase). The antibiotics often have a single site or mechanism of action, at least for low concentrations. This assumption is legitimate for penicillin, which is an extreme case of a non-competitive inhibitor (Harold, 1972) and which acts at the extracellular site of the membrane. If there exists a coordinated synthesis of CL and peptidoglycan or other cellular components, the regulation of the reactions must be disturbed by penicillin. Bacitracin has a quite different effect on lipid metabolism, possibly consisting of an effect on the integrity of the membrane. After 3.5 h of incubation with bacitracin the phosphogalactolipid content is increased, in comparison to the initial cellular amount, but not the content of the other phosphate-containing lipds (Table XVI) Apparently, the inhibition of the phosphogalactolipidsynthesis is less than that of the inhibition of overall growth. Inhibition of growth in the presence of chloramphenicol (75% of the control) results in a two-fold increase in the CL-content. In *B.amyloliquefaciens* the level of all phospholipids is decreased in the presence of chloramphenicol but no selective reduction has been demonstrated. The glycolipid content is also little affected in this organism (Glenn and Gould, 1973). In *S.aureus* lysyl-PG is decreased with a concomitant increase of PG in the presence of chloramphenicol, while the CL content is unchanged (Redai et al, 1972). In *B.bifidum* var.*pennsylvanicus* in the presence of streptomycin the only effect on the phospholipids is a decrease in the CL-content, while no significant effect on lipid metabolism is observed in the presence of actinomycin-D.

Although lipid synthesis is genetically controlled, environmental factors may vary the content and composition of the lipid complement by changing the activities of the enzymes involved or by changing the rates of enzyme synthesis. The synthesis of alcohol dehydrogenase in *E.coli* at unfavourable pH illustrates the interaction of the environment with the regulatory mechanisms of the cell (Pardee, 1961). Cultivation of this organism at pH 8 results in a decrease of the activity of this enzyme to one fourth of the value at pH 5, but the amount of this enzyme is 2.5 fold increased.

Little is known about the regulatory mechanisms of lipid synthesis. Merlie and Pizer (1973) claim to have shown a strict correlation between guanosine tetraphosphate accumulation and inhibition of phospholipid synthesis in *E.coli*. This nucleotide appears in both *E.coli* and in *B.subtilis*

(Swanton and Edlin, 1972) after cessation of RNA synthesis. Lipid synthesis will moreover be controlled to some extend by the compartmentalization of the enzymic machinery of the membrane. It has also been suggested that lipid incorporation into the membrane is loosely controlled by the protein incorporation into the membrane (Mindich, 1970). Further investigations will be required for a proper understanding of the environmental effects on the regulation of lipid metabolism in bacteria.

The changes in the amounts of glycerophosphorylgalactosyldiglyceride and its lyso-derivative in comparison with phosphatidylglycerol, cardiolipin and its lyso-derivatives for various conditions have been described in this chapter. In the next chapter the rates of synthesis and breakdown of the individual phospholipids will be compared in order to obtain some insight in their metabolic stability.

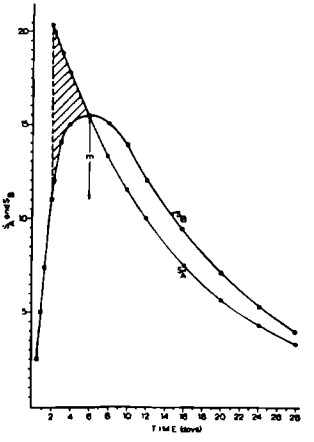

Figure 24
Specific activities of precursor A and product B plotted against time (from Zilversmit, 1960).

DYNAMIC STATE OF THE PHOSPHATE-CONTAINING LIPIDS

5.1. Introduction.

In the preceeding chapter we have used the ^{32}Pi information in the lipids as an index of the amounts of lipid phosphorus. The change in the amount of ^{32}Pi incorporated into the phospholipids in a certain time interval has been considered to be a measure of their net synthesis or net breakdown. This is approximately true, when the increases in the specific activity values of the lipids are small in the given time interval. After administration of ^{32}Pi to a culture the specific activities of the phospholipids increase to the isotope equilibrium value, i.e. to the specific activity value of the medium ^{32}Pi. The initial rate of incorporation corresponds to the real rate of synthesis which may be much higher than the rate of net synthesis. In all cells phospholipids appear to be synthesized at a rate well above the demands of growth (Dawson, 1966).

In this chapter we want to investigate the ^{32}P-incorporation in the phospho(galacto)lipids in the first hours after ^{32}Pi administration, in order to obtain insight into the dynamic state of these lipids. When the pool sizes of the phospholipids are comparable, the increase of the specific activity during incorporation is highest in the metabolically most active compound. When the bacteria are cultivated for some time in the presence of ^{32}Pi followed by a period of cultivation in a non-radioactive medium, the incorporated ^{32}P-radioactivity is chased most rapidly from the metabolically active compounds, while the radioactivity is largely retained in the metabolically stable compounds. Short and White (1971) found in their pulse-chase experiments with *S. aureus* that half of the incorporated ^{32}P-radioactivity in PG and CL is chased during exponential growth in non-radioactive medium in 0.5 and 1.4 bacterial duplications, respectively (one duplication in 30 min). The turnover of the phosphate moiety, i.e. the rate of synthesis and breakdown or conversion of the phospholipids, is higher in PG and CL than the turnover of the glycerol moiety of these molecules. The half-life of the incorporated [^{14}C]-glycerol in PG and CL amounts to 3.0 and 1.8 duplications, respectively, during the chase period.

The incorporated radioactivity during pulse-labeling need not be homogeneously distributed over the molecules. In *S. aureus* (Short and White, 1971) and in *E. coli* (Ballesta et al, 1971), the acylated glycerols of PG and CL are labeled by [^{14}C]-glycerol at a slower rate than the non-acylated glycerols. Similarly, the two phosphate moieties of CL are labeled at different rates. Short and White (1971) hydrolyzed CL after pulse-labeling with ^{32}P to PG and PA (phosphatidic acid) with CL-specific phospholipase D from *H.parainfluenzae*. The specific activity of the ^{32}P in the PA part of the molecule appeared to be twice that of the PG part. Their explanation was that the PG part of the molecule originates from a pool of PG with low specific activity and that the PA part comes from a PG pool with high specific activity. Two molecules of PG, each originating from a different pool, would combine to form CL. The existence of more than one metabolic pool of a certain phospholipid has been demonstrated also in *H.parainfluenzae* (Tucker and White, 1971), in *E.coli* (Ballesta et al, 1973) and in *B.stearothermophilus* (Card, 1973).

When overall-growth is inhibited, turnover of membrane components still occurs, though sometimes at a reduced rate. Card (1973) reported a reduced

turnover rate of the phosphate moieties of PG and CL after inhibition by chloramphenicol in *B.stearothermophilus*.

Kahane and Razin (1969) investigated the turnover of membrane proteins and membrane lipids of *M.laidlawii* after incorporation of [^{14}C]-phenylalanine and [^{3}H]-oleic acid. In cells harvested after 14-16 h of growth the turnover of the lipids became apparent only after a lag period, while the turnover of membrane protein commenced immediately. In cells from a 24 h culture the turnover rates of total membrane proteins and lipids were similar.

The dynamic state of a compound can be defined by its fractional turnover rate (k) and its turnover time (T_t). Both parameters, which are defined below, are unequivocal only in a steady state, i.e. when the concentration of the compound remains constant (Zilversmit, 1960). The phospholipids are in the steady state, when the synthesis of the phospholipids is balanced by breakdown, conversion or excretion from the cells. The fractional turnover rate (k) represents the fraction of a compound which is turned over, i.e. renewed, per unit of time. The turnover time is the average life time of the molecules of a compound. Fractional turnover rate and turnover time are related according to the furmula: $k = T_t^{-1}$. Fractional turnover rates can be determined from the decay of specific activity values during the chase period. Furthermore, the course of the specific activity curves of the individual compounds can give information about precursor-product relationships (Zilversmit, 1960).

When a compound A of a metabolic pathway is converted to a compound B, A is called the precursor of B. Assume that both compounds are labeled after ^{32}Pi incorporation, then incorporation into A is a prerequisite for the transfer of the ^{32}P-phosphate moiety from A to B. This implies, that the specific activity of A during ^{32}Pi incorporation is higher or equal to that of B from the moment that ^{32}Pi incorporation starts. When ^{32}Pi is removed after some time of incorporation then the radioactive phosphate moiety will be replaced by a non-radioactive one first in the precursor A and then in the product B. During the chase of the incorporated radioactivity from A and B the incorporation of ^{32}P-radioactivity in B is still continued for some time as a result of the renewal of its precursor A. The specific activity of B increases for some time, when that of A decreases. However, the specific activity of a product can never surpass that of its precursor. The specific activity curves of A and B cross when B reaches its maximum (Fig. 24). At the moment when the value of A equals that ofB the replacement of the phosphate moiety of B by non-radioactive phosphate becomes predominant, which results in a decrease of the specific activity of B. When the turnover rate of B is high in comparison to that of A the differences in the specific activity values of A and B will be small both during the pulse period and during the chase period. The same holds true, when the rate of turnover of A and B are comparable, but when the pool size of B is very small compared to that of A. In that case the fraction of the molecules of B that is turned over per unit of time is high in comparison to that of A. The fractional turnover rate (k) of B can be calculated by grafical means from the specific activity curves (Zilversmit, 1960).

For the calculation of k we must be sure that the following four conditions are fulfilled.

(i) B is in steady state,
(ii) the turnover rates of precursor A and product B remain constant though they need not be equal during the period of the experiment,
(iii) reutilization of non-metabolized residual radioactive precursors and breakdown products of the metabolic pathway is negligible,
(iv) the newly formed molecules of A and B which are introduced in the

given compartments are completely mixed with the "old" molecules i.e. there exists only one metabolic pool of A and of B.

Our attempt to investigate the possible metabolic relationship between the phosphogalactolipid and phosphatidylglycerol or cardiolipin was prompted by the finding of Pieringer (1972) that both PG and CL can function as a phosphate donor for the synthesis of a phosphoglycolipid. In a cell-free system the synthesis of phosphatidyldiglucosyldiglyceride is achieved by a transfer of the phosphatidylgroup of either PG or CL to diglucosyl diglyceride. This reaction is catalyzed by a disrupted membrane enzyme preparation from *Streptococcus faecalis*. In our experiments we have not investigated the precursor-product relationship by means of an *in vitro* enzymic reaction, but we have followed the incorporation and chase of radioactivity *in vivo*.

5.2. Methods.

The dynamic state of the phospholipids is studies in the following way. The specific activities of the phospholipids are determined after a certain time of $^{32}P_i$ incorporation The specific activity values are followed, when radioactive medium is replaced by non-radioactive medium after a 60 min pulse. The amounts of radioactivity retained by the phospholipids during the chase period are also determined. $^{32}P_i$ is incorporated into the phospholipids for 60 min in medium of normal specific activity (1400 cpm/nmole P), after which time the specific activity of $^{32}P_i$ in the medium is decreased 10-fold by addition of K_2HPO_4.

^{32}P-phosphate is added to all cultures in the amount of 1 mCi/l culture (section 2.2). In the pulse-chase experiments $^{32}P_i$ is added after 10.5 h of cultivation under normal conditions and growth is continued for 60 min. The pulse-labeled cells are then harvested by centrifugation (10 min at 13.000 g) and subsequently resuspended the same volume of non-radioactive medium as before. The chase period is started after prewarming of the culture to 37 °C. Details of the other experiments are explained in the legends to the figures.

The relative amounts of ^{32}P-radioactivity in the individual phospholipids are determined after separation of the lipids by two-dimensional thin-layer chromatography (section 2 5). Preparative one-dimensional chromatography on silicagel HR is applied in order to determine the specific activities of PG, compound-15 and CL.

Chloroform-methanol-acetic acid-water (125 : 37 : 9.5 . 1 5, by vol.) is used as developer in one dimensional chromatography.

Compound-15 when contaminated with lyso-CL (see Fig. 7), is chromatographed once again one-dimensionally with chloroform-methanol-7M ammonia (60 : 35 : 5, by vol.). The phospholipids are located on the chromatogram by radioactivity scanning (section 2.4.). After elution of the phospholipids from the silicagel, samples are taken for ^{32}P counting and phosphorus analysis. The amount of lipid phosphorus is calculated for each phospholipid as the quotient of ^{32}P-radioactivity and its specific activity.

5.3.1. Isotope equilibrium between cells and medium.

After administration of $^{32}P_i$ to a culture incorporation of the isotope in all phosphate-containing cellular components will continue until the specific activity values of these compounds are equal to that of $^{32}P_i$ in the growth medium. The maximum level of incorporation is the same for all phosphate compounds, but the time required for isotope equilibrium may vary significantly for the various compounds. The ^{32}P-incorporation data are always standardized for an administration of exactly 1 µCi $^{32}P_i$ per ml culture. Phosphorus assay reveals that the total phosphate pool of the medium is 2.58 mM, of which 1.44 mM originates from K_2HPO_4. The difference between the total amount of medium phosphate and K_2HPO_4, must be derived from other nutrients, mainly from casein-hydrolyzate (see section 2.1).

The specific activity of the total medium phosphate pool is 850 cpm/nmole P in the presence of 1 µCi $^{32}P_i$, added as tracer. However, $^{32}P_i$ incorporation into the phospholipids after 6 bacterial duplications gives a specific activity value for the total lipid fraction of about 1400 cpm/nmole P, which is much higher than expected on the basis of the specific activity of the medium. This implies that the contribution of the nutrients to the phosphate pool (1.14 mM) must consist mostly of phosphate containing compounds that are not taken up into the cells and used for phospholipid metabolism. If K_2HPO_4 would function as the only phosphate source that can pass the membrane, the isotope equilibrium value of the phospholipids would be 1530 cpm/mole P, which is indeed close to the observed value.

5.3.2. Turnover of the phospholipids studied by incorporation experiments.

After cultivation of the organism under normal growth condition for 10.5 h, $^{32}P_i$ is added and its incorporation into the phospholipids is followed for 5 h. During this time interval growth continues from the late log phase to the stationary phase (Fig. 25a), while the specific activity of the total lipid fraction increases linearly (Fig. 25b). The specific activity value does not level off to a value close to that of the total medium phosphate pool (850 cpm/nmole P). This shows that the isotope equilibrium value is much higher as has been demonstrated above (section 5.3.1). Apparently, ^{32}P-incorporation into the phospholipids is not affected by the decreasing rate of overall growth, which suggests that the rate of phospholipid synthesis greatly exceeds the rate of net synthesis of the phospholipids.

The contributions of turnover and of net synthesis of the phospholipids to the ^{32}P-incorporation during the first hours after isotope addition has been estimated as follows. Between times 10.5 and 13.5 h the specific activity of the total phospholipid fraction increases from 0 to 440 cpm/nmole P (Fig. 25b). In the same time a net synthesis of phospholipids corresponding to 7 nmoles/ml culture has been observed. This amount of net synthesis corresponds to an incorporation of about 1500 cpm/nmole P, based on the average specific activity value of 220 cpm/nmole P. However, the real amount of ^{32}P-incorporation in this time interval amounts to 9300 cpm/ml. This means that the rate at which "old" molecules are replaced by "new" ones, i.e. the turnover rate, is much higher than the rate of net synthesis. The contribution of turnover to the total ^{32}P-incorporation amounts to 9300-1500 = 7500 cpm/ml, which is 5 times as high as that due to the net synthesis. This shows that the phospholipids in the late log-phase are

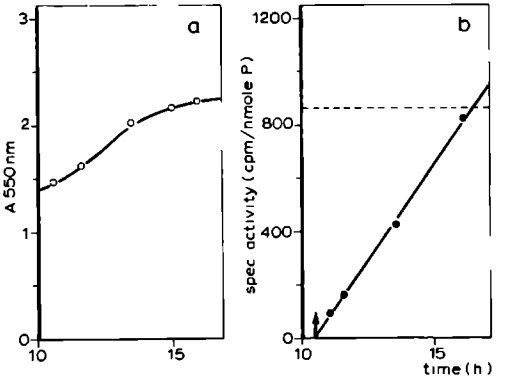

Figure 25
Specific activity of the total lipid fraction when $^{32}P_i$ is added after 10.5 h of growth. The culture is grown under normal condition at non-constant pH. Arrow indicates addition of isotope. The interrupted line represents the specific activity of the total phosphate pool of the medium.

still in a dynamic state.

In this experiment the half-equilibrium value of the specific activity (700 cpm/nmole P) is achieved in 5 h, but this time period is markedly shortened by resuspending the cells in fresh medium before supplying the isotope (Table XVII).

The effect of growth inhibitors is studied by determining the specific activity of the total lipid fraction after 2.5 h of ^{32}P incorporation in the presence of chloramphenicol, penicillin and bacitracin (experiment A, Table XVII). The ^{32}P-incorporation in the presence of chloramphenicol and penicillin occurs at about the same rate as in the control culture as appears from the specific activity values (600 and 650 cpm/nmole P). In these cultures growth is moderately inhibited in the presence of chloramphenicol (0.85 duplication) and strongly inhibited in the presence of penicillin (0.29 duplication). This means that the dynamic state of the phospholipids, i.e. their rate of synthesis and breakdown, is not changed, while overall growth is partly inhibited by these antibiotics. On the other hand, the rate of ^{32}P-incorporation into the phospholipids in the presence of bacitracin is about half of that in the control culture, as appears from the specific activity value.

In a similar experiment the effects of absence of human milk and of a slightly alkaline medium pH (pH 8.0) on phospholipid metabolism are studied (Experiment B, Table XVII). After 3.5 h of ^{32}Pi incorporation the specific activity of the control culture amounted to 1100 cpm/nmole P. The inhibition of growth under theseconditions is accompanied by a decreased rate of phospholipid synthesis as appears from the decreased values of specific activity.

The specific activity of the total lipid fraction is an average of the specific activities of the individual phospholipids, which may vary quite markedly. The values for PG, compound-15 and CL of experiment B (Table XVII) are shown in Fig. 26. The specific activity of PG is higher than that of CL in these cultures, which is consistent with the precursor-product relationship between both phospholipids. The level of ^{32}P-incorporation in both PG and CL decreases parallel to the decrease in overall growth of the cells.

Table XVII

Specific activity values of the total lipid fraction after 2.5 h and 3.5 h of $^{32}P_i$ incorporation under different growth conditions.

In experiment A cells of a 10.5 h culture are used as inoculum after centrifugation and resuspension in fresh medium. Similarly, cells of a 16 h culture are used as inoculum in experiment B. Data of experiment B are obtained from Fig. 26.

Experiment	Condition	^{32}P incorporation	Growth (doublings)	Spec. activ. (cpm/nmole P)
A	control	2.5 h	1.00	610
	chloramphenicol 60 µg/ml	2.5 h	0.85	600
	penicillin 2 µg/ml	2.5 h	0.29	650
	bacitracin 400 µg/ml	2.5 h	0.59	330
B	control	3.5 h	1.0	1100
	without human milk	3.5 h	0.6	870
	pH 8.0	3.5 h	0.2	510

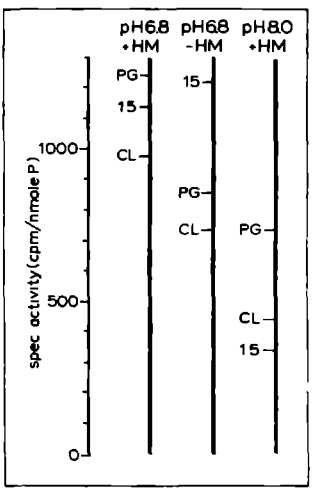

Figure 26

Differences in metabolic activity of the main phospholipids under different conditions of growth.

Bifidobacterium bifidum var. *pennsylvanicus* is cultivates in the presence and in the absence of human milk (HM) at a non-constant pH for 3.5 h in the presence of $^{32}P_i$. The culturesare pH 6.8 or 8.0 at the moment of $^{32}P_i$ administration. The relative amounts of lipid-^{32}P and lipid-phosphorus of the individual phospholipids are given in Fig. 13.

In the control culture (pH 6.8 + HM) the specific activity of PG is close to the isotope equilibrium value (1400 cpm/nmole P) after 3.5 h of ^{32}Pi incorporation. Compound-15 is in a metabolically active state comparable to that of PG in the control culture (pH 6.8 + HM), as shown by the similarity of their specific activities. The metabolic activity of compound-15 under the two growth inhibitory conditions (pH 6.8 - HM and pH 8.0 + HM) changes markedly. In a medium devoid of human milk the ^{32}P-incorporation into compound-15 is much larger than that into PG, the specific activity of compound-15 being close to isotope equilibrium. At pH 8.0 compound-15 is metabolically more stable than CL. Apparently, the ^{32}P-incorporation into compound-15 is little affected when cell wall synthesis is inhibited by lack of human milk, in contrast to the strong inhibition of ^{32}P-incorporation at alkaline pH (8.0). This suggests, that the rate of turnover of the phosphogalactolipid depends on the extracellular environment in a different way than is the case for the turnover rates of PG and CL.

5.3.3. Pulse-chase experiments.

Pulse-chase experiments have been done primarily to determine the existence of precursor-product relationships between compound-15 and PG or CL. Cells grown to the late log phase (10.5 h) are pulse-labeled in the next 60 min, while growth continues for 0.1 bacterial duplication. After the pulse period the specific activities of PG, compound-15 and CL amount to 275, 95 and 35 cpm/nmole P, respectively. The labeled cells are then centrifuged and resuspended in fresh non-radioactive medium and cultivated again. During the chase period growth continues for 0.9 duplication in 3.5 h (Fig. 27a), resulting in a drop of the pH of the medium from 6.8 to 5.4 through the

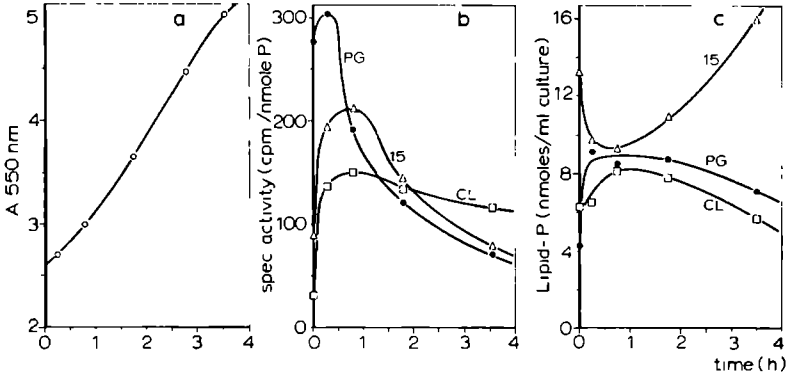

Figure 27

Chase of lipid-^{32}P during rapid growth. After cultivation for 10.5 h $^{32}P_i$ is supplied (1 µCi/ml culture) and then cultivation is continued for 60 min. The cells are then sedimented by centrifugation , resuspended in fresh non-radioactive medium (pH 6.8 with human milk) and cultivated again for 3.5 h at non-constant pH.

a) Optical density during the chase period.

b) Specific activity of phosphatidylglycerol (PG), compound 15 and cardiolipin (CL) during chase period.

c) Pool size of the phospholipids.

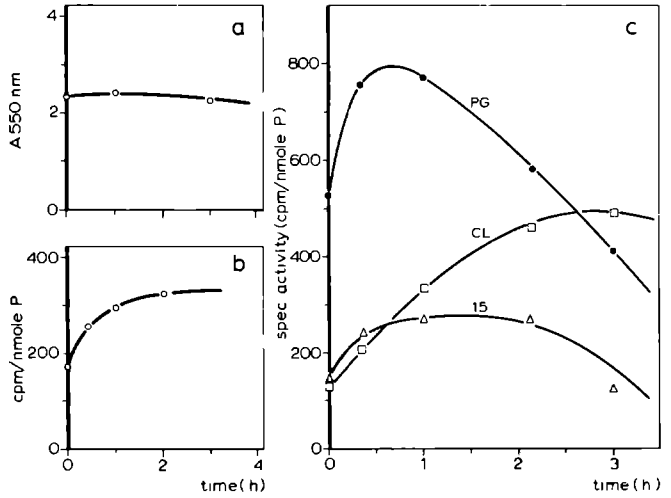

Figure 28

Chase of lipid-^{32}P in the presence of penicillin. Cells are pulse-labeled as described in Fig. 27 and are subsequently cultivated in non-radioactive medium of pH 5.5 containing penicillin (10 µg/ml, 500 U/mg).

a) Optical density during the chase period.

b) Specific activity of the total lipids during the chase period.

c) Specific activity of the individual phospholipids.

excretion of acid metabolites.

The changes in the specific activities of PG, compound-15 and CL during the chase period are shown in Fig. 27b. The specific activity of compound-15 reaches a maximum, when the curves of PG and compound-15 intersect. This suggests that PG is a direct precursor of compound-15 rather than of CL under these conditions. The specific activity of PG increases during the first 30 min of the chase period. This means, that the specific activities of its precursors (Pi, glycerophosphate, phosphatidic acid, phosphatidylglycerophosphate, see Fig. 3) must be higher than that of PG during this period.

During the first hour a net synthesis of CL is observed which is accompanied by a rapid increase in its specific activity (Fig. 27b and c). Thereafter the total amount of CL slowly decreases, while its specific activity decreases only slightly. This suggests that during the chase period PG is initially converted mainly to CL, but after 1 h PG is used mainly for the synthesis of compound-15.

During the chase period rapid growth occurs, while the amount of CL decreases slowly. This results in a decrease of the cellular amount of CL (see also Table XVI). A high CL content is characteristic for the stationary phase (Table XIII).

The turnover time of PG can be calculated for the period in which steady state exists. During steady state (between times 0.5 and 2.5 h) the specific activity of PG decreases exponentially with a half-life of about 90 min. This value must be multiplied by a factor 1.44 to yield the turnover time (Zilversmit, 1960). However, this turnover time of 90 x 1.44 = 130 min represents a maximum value. The average life time of the PG molecules may be

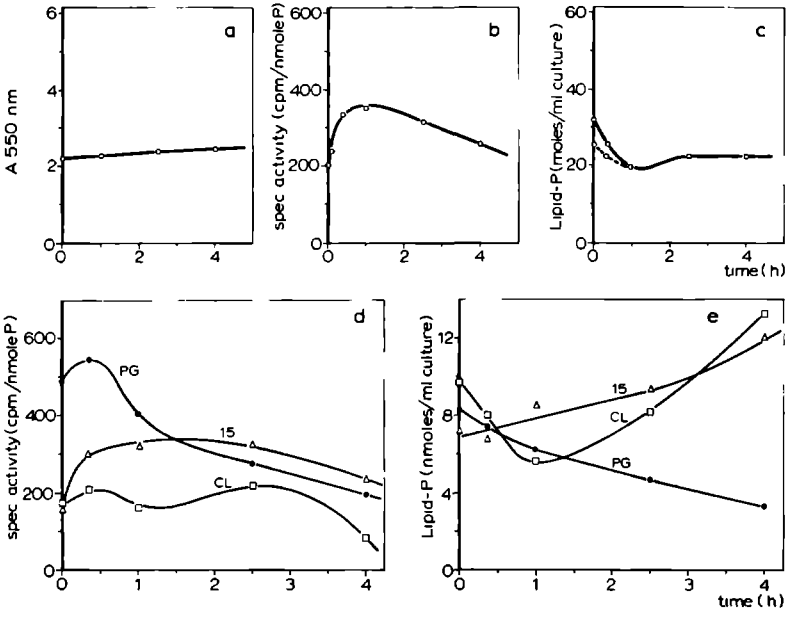

Figure 29

Chase of lipid-^{32}P in the presence of bacitracin. Cells are pulse-labeled as given in Fig. 27 and subsequently cultivated in non-radioactive medium of pH 5.5 containing bacitracin (400 µg/ml, 20 U/mg).

a) Optical density during the chase period.

b) Specific activity of the total lipid fraction during the chase period.

c) Pool size of the total lipid fraction. The interrupted line represents the sum of PG + CL + compound 15.

d) Specific activities of PG, compound 15 and CL.

e) Pool sizes of PG, compound 15 and CL.

considerably shorter, when the specific activity of intracellular Pi approaches that of PG during the period of steady state.

This pulse-chase experiment has been repeated with the only difference that growth is inhibited during the chase period by addition of penicillin, bacitracin or chloramphenicol or by lack of human milk. We hoped that compound-15 would approach the steady state under some of these growth inhibitory conditions in order to establish the precursor-product relationship between PG and compound-15. Steady state of compound-15 is a prerequisite for the calculation of its turnover time.

In the 60 min pulse period prior to chase in the presence of penicillin the culture has grown for 0.20 duplication and the specific activity values of PG, compound-15 and CL amount to 530, 140 and 140 cpm/nmole P, respectively. During the chase period overall growth is fully inhibited (Fig. 28a). The specific activity of the total lipid fraction increases for 2 h to a value of 330 cpm/nmole P (Fig. 28b), owing to the high specific activity of the intracellular ^{32}Pi. The specific activity curves of PG, CL and compound-15 are given in Fig. 28c. These curves suggest that PG is mainly converted to CL in the presence of penicillin. In chapter 4 (Table XVI) we have reported that the cellular amount of CL is increased 6-fold in 3.5 h by the

action of penicillin, which implies a high rate of net synthesis of CL. The content of compound-15 remains constant in the presence of penicillin.

In the 60 min pulse period prior to chase in the presence of bacitracin and chloramphenicol the cultures have grown for 0.25 and 0.20 bacterial duplications, respectively. The specific activity values in both cultures amount to 490 and 550 for PG, to 170 and 170 for compound-15, and to 180 and 190 cpm/nmole P for CL, respectively. During the chase period there is hardly any growth in the presence of bacitracin (Fig. 29a), while a small increase in optical density is observed in the presence of chloramphenicol (Fig. 30a). The maximum values of the specific activity of the total lipid fraction in both cultures are comparable to the maximal value in the presence of penicillin (Figs. 28b, 29b, 30b), but they are reached faster (after 1 h). The increases in the specific activity of the total lipid fraction during the preceding 60 min pulses occur at a much faster rate than the decreases during the chase periods. This means that the intracellular pool of ^{32}Pi is slowly equilibrating with the extracellular pool of non-radioactive inorganic phosphate.

The total amount of lipid phosphorus decreases during the first hour, which must be due to a decrease in the amount of CL (Fig. 29c and e, 30c and e). Lyso-CL and Lyso-compound 15 account for a small fraction of the lipid-phosphorus. In the presence of chloramphenicol they constitute 15-20% of the total lipidphosphorus during the whole chase period (Fig. 30c), while in

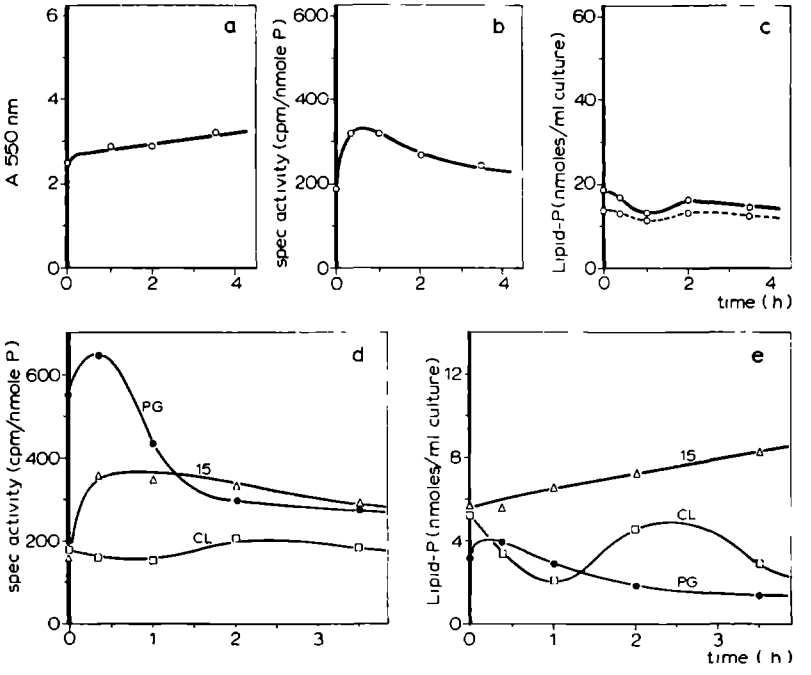

Figure 30
Chase of lipid-^{32}P in the presence of chloramphenicol. Procedure described in legend to Fig. 29. Chloramphenicol is present in a concentration of 60 µg/ml.

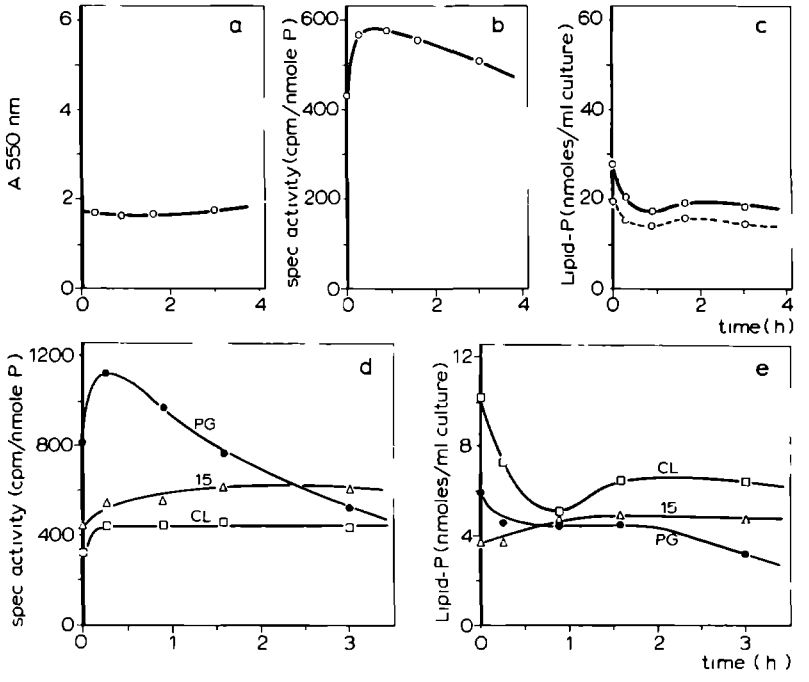

Figure 31
Chase of lipid-^{32}P in the absence of human milk. Procedure described in legend to Fig. 29.

the presence of bacitracin they are detectable only during the first hour (Fig. 29c).

The specific activity curves of PG, compound-15 and CL are comparable in both cultures (Figs. 29d and 30d). The specific activity of compound-15 rapidly increases during the first 30 min and then remains rather constant. This suggests that the turnover time of compound-15 is not constant during the chase period in the presence of bacitracin or chloramphenicol. Furthermore, the conversion of PG to compound-15, which may occur under these conditions, is disturbed by the fact that compound-15 is not in the steady state. In the presence of bacitracin compound-15 increases from 7 to 12 nmoles/ml culture in 4 h (Fig. 29c), while an increase from 5 to 8 nmoles/ml is observed in the presence of chloramphenicol. In the presence of bacitracin the specific activity of CL is rather constant during the first 3 h and then decreases. This decrease is accompanied by a significant net synthesis of CL (Fig. 29d and e). In the presence of chloramphenicol the specific activity of CL is nearly constant for 4 h, while the amount of CL is not constant (Fig. 39d and e).

Chase of incorporated radioactivity has also been studied in the absence of human milk. During the preceding pulse labeling of the cells for 60 min, growth continues for 0.55 duplication, while in the pulse-chase experiments described above growth continues for only 0.20-0.25 bacterial duplication. The specific activities of PG, compound-15 and CL amount to 810, 460 and 330 cpm/nmole P, respectively. During the chase period overall growth is

fully inhibited by lack of human milk (Fig. 31a). The specific activity curves of the total lipid fraction and those of PG, compound-15 and CL are similar to those found in the presence of bacitracin and of chloramphenicol (Figs. 29b and d, 39b and d, 31b and d). However, the values are about twice as high in the culture devoid of human milk, which is proportional to the stimulated overall growth of the cells during the pulse period. The decrease in total lipid phosphorus during the first hour of the chase period is also a result of the decrease in CL in this period. After 1 h the pool sizes of PG, compound-15 and CL are rather constant.

Although steady state of compound-15 is better approached than in the pulse-chase experiments above, this experiment yields no further evidence for a precursor-product relationship between PG and compound-15. Since the pool sizes of both lipids are comparable in this experiment, we would expect that chase of radioactivity from PG would be accompanied by a significant change in the specific activity of compound-15, which does not occur.

Change in the specific activity value of compound-15 does not necessarily reflect that turnover occurs when steady state is not achieved. For instance, when the rate of breakdown or conversion of compound-15 is small compared to the rate of synthesis, the specific activity of the total pool decreases during the chase period. The absence from turnover then appears from the incorporated amount of radioactivity, which is retained when the

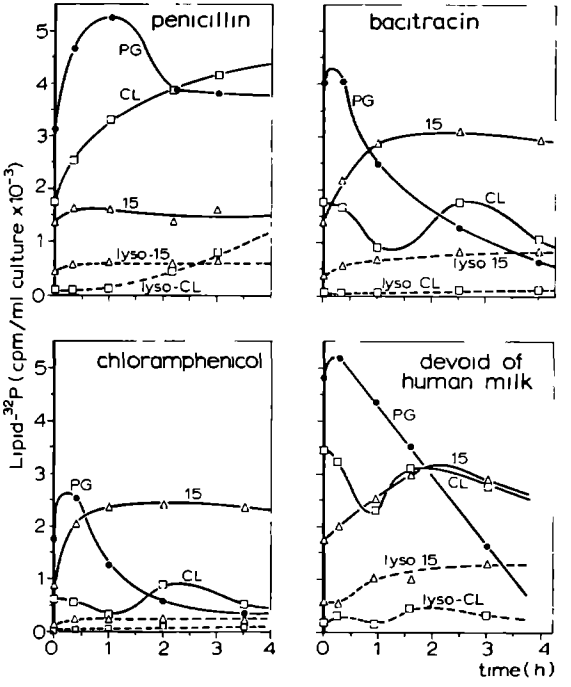

Figure 32

Radioactivity retained in the individual phospholipids during chase period under growth-inhibitory conditions. The corresponding specific activity values and the pool sizes of the phospholipids are given in Figs. 28 to 31.

molecules, once formed, are metabolically stable. This is the case after one hour of incubation in the presence of bacitracin (Fig. 29d and e, Fig. 32).

The radioactivity curves of the other pulse-chase experiments described above are also given in Fig. 32. In the presence of penicillin no turnover of compound-15 occurs. All radioactivity incorporated during the pulse period is retained during the chase period. In the presence of bacitracin chase of radioactivity from PG is accompanied by a continued incorporation of ^{32}P radioactivity into compound-15 in agreement with a conversion

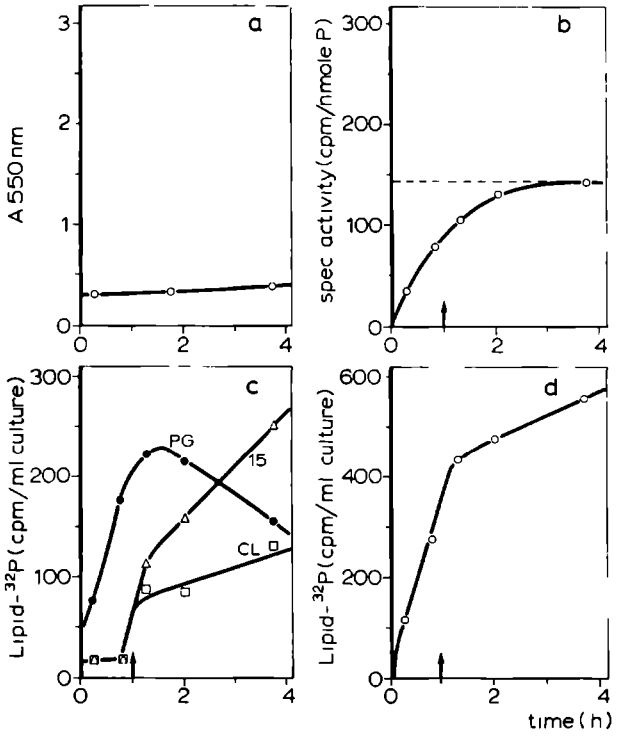

Figure 33

Chase of lipid-^{32}P from a culture devoid of human milk by addition of non-radioactive phosphate. A 16 h culture is used as inoculum (10^{4}, by vol.). ^{32}Pi is supplied to the medium without human milk at t = 0 h. After 60 min of ^{32}P incorporation the concentration of K_2HPO_4 of the medium is raised from 0.25 to 2.5 g/l, indicated by the arrow. The pH of the culture has a constant value of 5.5. The total amount of radioactivity in the culture remains constant during the experiment.

a) Optical density.

b) Specific activity of the total lipid fraction. The interrupted line is the equilibrium value after addition of K_2HPO_4.

c) Radioactivities of PG, compound 15 and CL before and after phosphate addition.

d) Radioactivity of the total lipid fraction before and after phosphate addition.

of PG to compound-15. However, the amount of radioactivity in compound-15 remains constant after about 1 h, while the loss of radioactivity from PG continues. The same holds true when growth is inhibited by chloramphenicol. Probably, the rate of turnover of compound-15 decreases during cultivation in the presence of bacitracin and chloramphenicol so that the radioactivity, once incorporated, is retained. The continued loss of radioactivity from PG may be explained by deacylation or by conversion of PG to a compound which is not recovered in the total lipid fraction. In the culture devoid of human milk the radioactivity of PG is chased at a constant rate, while the amount of radioactivity increases slowly and then decreases. The symmetric course of the curve of compound-15 suggests that turnover of compound-15 occurs under this condition, but the chase period has been too short to establish it. At any rate the disappearance of radioactive molecules of PG can be ascribed only to a small extent to a conversion of PG to compound-15, since the change in the specific activity of compound-15 is very small (Fig. 31d).

5.3.4. Effect of lowering the specific activity of ^{32}Pi by addition of K_2HPO_4.

A 16 h culture is added as inoculum (10%, by vol.) to a medium devoid of human milk, and ^{32}Pi is added simultaneously. Overall growth is inhibited through lack of human milk (Fig. 33a). The culture pH of 5.5 remained constant during cultivation. The concentration of K_2HPO_4, which is 0.25 g/l in all cultures used for ^{32}Pi incorporation (section 2.2), is raised after 60 min from 0.25 to 2.5 g/l (indicated by the arrows in Fig. 33). This lowers the specific activity of ^{32}Pi from 1400 to 140 cpm/nmole P.

The specific activity of the total lipid fraction amounts to 90 cpm/nmole P after 60 min, while the isotope equilibrium value up to this point is 1400 cpm/nmole P. This means, that in the absence of human milk isotope equilibrium is obtained only after several hours. Lowering of the specific activity of ^{32}Pi to 140 cpm/nmole P by addition of K_2HPO_4 accelerates the isotope equilibrium between ^{32}Pi and the phosphate containing lipids, as appears from the specific activity curve for the total lipid fraction (Fig. 33b).

The initial rate of ^{32}Pi incorporation amounts to about 350 cpm/ml culture/hour, which value is lowered to about 50 cpm/ml/hour after addition of K_2HPO_4 (Fig. 33d). Turnover will contribute predominantly to ^{32}P-incorporation during the first hour, while net synthesis will prevail between 2 and 4 h when isotope equilibrium is nearly achieved.

The specific activity curve for the total lipid fraction gives at any moment the mean value of those of the individual phospholipids. The initial increase in the specific activities of PG, compound-15 and CL may vary significantly (see Fig. 26). However, the maximal values for all phospholipids will be equal, although the time required for the establishment of isotope equilibrium will be different for the individual phospholipids.

Specific activity curves of the individual phospholipids could not be determined in this experiment, since not enough cells were available. The radioactivity curves of PG, compound-15 and CL are given in Fig. 33c. During the first hour rapid ^{32}P-incorporation occurs into PG, but not in compound-15 and CL. After addition of K_2HPO_4 the amount of radioactivity in PG decreases, which decrease is accompanied by an increase of the radioactivity in compound-15 and to a smaller extent in CL.

The specific activity of compound-15 and of CL must be very low during the first hour. The value for the total lipid fraction still amounts to 90 cpm/nmole P after 1 h. This means that the specific activity of PG at this

moment must be far above this value, since the pool size for PG is not larger than the pool sizes for compound-15 and of CL at pH 5.5 (see Fig. 31e).

At the moment of K_2HPO_4 addition the specific activity of PG must be higher than the final equilibrium value (140 cpm/nmole P), so that radioactivity will be chased from PG until isotope equilibrium is reached. Conversion of PG to compound-15 during this period is suggested by the continued isotope incorporation into compound-15, just as is found in the culture devoid of human milk in which the radioactive medium is replaced by non-radioactive medium (Fig. 32).

5.4. Discussion.

The dynamic state of the phospholipids can be quantitatively described by the fractional turnover rates of the phospholipids. The following conditions must be satisfied in order to permit calculation of the fractional turnover rate (k) of any compound: i) steady state must exist, ii) k must be constant, iii) recycling of precursors must be negligible and, iv) one homogeneous pool must be present.

We have seen that steady state of the phospholipids is difficult to achieve. The steady state of PG is approached, when cells grown to the late log phase are resuspended in fresh medium with and without human milk (Figs. 27c and 31e). In cultures without human milk compound-15 is also in the steady state (Fig. 31e). CL has never been found to be in the steady state under the investigated conditions. A rapid decrease of the amount of CL has been observed during the first hour after resuspension of the late log phase cells in a medium containing bacitracin or chloramphenicol or lacking human milk (Figs. 29e, 30e, 31e).

In the presence of bacitracin or chloramphenicol compound-15 is not in the steady state and also its fractional turnover rate is not constant. During the first hour after resuspension of the cells in fresh medium containing these antibiotics ^{32}P-incorporation is continued (Fig. 32), possibly through conversion of PG to compound-15. No significant net synthesis of compound-15 has taken place during the first hour (Figs. 29e and 30e). Therefore, the increase of incorporated radioactivity of compound-15 implies active turnover during the first hour. Thereafter compound-15 has become metabolically stable, since the incorporated radioactivity is retained (Fig. 32). A decrease of the rate of turnover in the presence of chloramphenicol has also been reported for PG and CL in *B.stearothermophilus* (Card, 1973).

The third condition, that recycling of the precursors is negligible, is hardly to check. It is clear that recycling of intracellular inorganic phosphate from ATP, sugar phosphates, or from RNA and DNA is quite likely. The equilibration of intracellular inorganic phosphate with K_2HPO_4 of the medium occurs very slowly, as appears from the continued ^{32}Pi incorporation into the total lipid fraction after resuspension of pulse-labeled cells in non-radioactive medium (Figs. 28b, 29b, 30b, 31b). It cannot be said whether the slow equilibration between intracellular and extracellular inorganic phosphate is mainly a result of recycling from cellular compounds or of a slow transport across the membrane. Excretion of solutes from cells usually involves carrier-mediated transport (section 1.6).

Considering the fourth condition that one homogeneous pool must be present, we have no experimental evidence for the existence of a heterogeneity of the PG pool or CL pool, as has been reported by Tucker and White (1971), by Ballesta et al. (1973) and by Card (1973). These complications hamper the

calculation of fractional turnover rates.

Half-life times are often used as a measure for turnover when the conditions mentioned above are not fulfilled. The half-time of a compound is the time in which half of the incorporated radioactivity is lost during a chase period. Radioactivity incorporated into PG during the pulse period is lost exponentially during rapid growth in the chase period (Fig. 27b). Half of the incorporated radioactivity is chased in 90 min, while growth continues for 0.5 bacterial duplication. Short and White (1971) reported also for PG a half-life time of 0.5 doubling (15 min) in *S.aureus* during rapid growth.

The pulse-chase experiments have been carried out in order to investigate the existence of a precursor-product relationship between PG of CL and compound-15. The specific activity curves of PG and compound-15 suggest that PG functions indeed as a precursor of compound-15 during rapid growth (Fig. 27b). When the radioactivity is chased in the presence of bacitracin or chloramphenicol or in the absence of human milk the specific activity curves of PG and compound-15 intersect, but the change in the specific activity of compound-15 is very small. This means that under these conditions PG is converted to compound-15 to a small extent. Chase of radioactivity from PG under these conditions probably implies a significant degradation of PG or a conversion to a compound which is not recovered in the total lipid fraction. The total ^{32}P-incorporation during the first hours in late log phase cells is about 6 times higher than is required for net synthesis of the phospholipids i.e. the total rate of synthesis under steady state is 5 times higher than the net rate of synthesis.

From the ^{32}P-incorporation studies it appears that isotope equilibrium is quickly reached when the cells grow at a fast rate. Growth of 0.25 and 0.55 duplication during ^{32}P-incorporation in the late log phase corresponds to a specific activity of the total lipid fraction of 200 and 430 cpm/nmole P respectively (Figs. 29b and 30b). The specific activities of the individual phospholipids after 0.25 duplication are also about half of the values after 0.55 duplication. This also shows that the equilibration between intracellular and extracellular inorganic phosphate is determined by the rate of influx, which is high during rapid growth. The initial amount of intracellular inorganic phosphate has little effect on the specific activity of ^{32}Pi in the cells after many duplications.

In the presence of penicillin a significant excretion of phosphate-containing compounds from the cells takes place (section 4.3.4). Excretion of intracellular inorganic phosphate is unlikely, since the specific activity of the total lipid fraction continues to increase for at least 2 h in the presence of penicillin after removal of ^{32}Pi from the medium (Fig. 28b). We have not characterized the excreted compounds, but it is possible that membrane phospholipids are involved. If phospholipids are indeed excreted, phospholipid synthesis must be high since not only are the excreted phospholipids replaced by new molecules but there is even a two-fold increase in the total amount of lipid phosphorus and a 6-fold increase of the cellular amount of CL (Table XVII). This can explain why the specific activity of the total phospholipid fraction increases in the presence of penicillin at a rate comparable to that in the non-inhibited control culture (Table XVII).

In this chapter we have shown that PG can probably be converted to compound-15, but conclusive evidence could not be obtained from the pulse-chase experiments. However, the conversion of PG to compound 15 has been recently confirmed *in vitro*, making use of an enzyme fraction of *B.bifidum* var. *pennsylvanicus* (Veerkamp, unpublished).

From the pulse-chase experiments it appears that PG is metabolically most active. PG is turned over under growth inhibitory conditions even when

compound-15 and CL have become metabolically stable. The rapid loss of radioactivity from PG during a chase period has been observed among other bacterial species such as *E.coli* (Ohki, 1972; Ballesta and Schaechter, 1973) and *B.stearothermophilus* (Card, 1973).

So far, the process of rapid turnover of the phospholipids is not very well understood. Rapid turnover probably reflects some activity of the membrane enzymes. A functional relation between PG metabolism and biological events in the cytoplasmic membrane has been studied in *E.coli*. Ohki (1972) has demonstrated in this bacterium that turnover of PG takes place in a stepwise manner at a specific point of cell duplication. The turnover of PG is accompanied by a stepwise increase of cytochrome b_1 in the membrane and a stepwise increase of L-α glycerophosphate accumulation, which presumably reflects an increase in the transport activity of the membrane.

For a better understanding of the biological significance of the turnover of PG in *B.bifidum* var.*pennsylvanicus* it will be useful to synchronize the bacterial duplications (section 1.8).

The phosphogalactolipid compound-15 studied in this thesis is described in the general introduction as a phospholipid of taxonomic significance for the differentiation between *Lactobacilli* and *Bifidobacteria*. Its structure has been fully elucidated for *B.bifidum* var.*pennsylvanicus* (Chapter 3). It can, however, not be excluded that the corresponding lipid in other *Bifidobacteria* only differ in the configuration of the glycerophosphate or of the galactose moiety. Phosphogalactolipid compound-15 constitutes a major component in *Bifidobacteria*, while phosphoglycolipids found in other bacteria are generally minor compounds except in *Mycoplasmas* (section 4.4).

The structure of compound-15 appears to be glycerophosphorylgalactosyldiglyceride with the glycerophosphate moiety in the *sn*-1 configuration, the glycosidic bond in the β-configuration and galactose mainly in the furanose form. The core structure, glycerophosphorylgalactosylglycerol, has been elucidated by quantitative analysis and by the analysis of the products of strong alkaline hydrolysis. These products consist of glycerol, glycerophosphate, galactosylglycerol and phosphorylgalactosylglycerol, the phosphate-containing products being overlap fragments. The furanose and pyranose isomers of galactose made up 80% and 20%, respectively of galactosylglycerol.

The appearance of phosphorylgalactosylglycerol might suggest that galactose in this fragment is present merely in the furanose configuration, but this proves not to be true. The occurrence of phosphorylgalactosylglycerol with galactose partly in the pyranose configuration shows that the formation of a phosphodiester intermediate between carbon atom 3 or 4 and carbon atom 6 of galactose is likely during hydrolysis of the phosphodiester bond. So far, phosphoglucolipids have been reported in the literature with glucose in the pyranose form only. From these compounds no product is released on alkaline hydrolysis in which the phosphateglucose linkage is retained.

Glycerophosphorylgalactosylglycerol has also been established as the core structure of lyso-derivative and of acylated derivative of compound-15, which are both present in *B.bifidum* var.*pennsylvanicus*, but the relative amounts of the furanose and the pyranose forms may differ from that in compound-15.

In chapter 4 we have studied the effect of some environmental factors on the phosphogalactolipids and on the other membrane phospholipids.

The synthesis of CL, lyso-CL, compound-15 and lyso-15 occurs at a constant rate during growth in contrast to the synthesis of PG. The initial net increase of PG changes gradually into a net decrease during growth. Therefore, the change in the amount of PG per ml culture dictates the changes in the relative amounts of the other phospholipids.

The decrease of the pH of the medium during growth from 6.8 to 5.2 by the fermentation of sugars has no significant effect on the phospholipid synthesis. When the initial pH of 6.8 is maintained by titration of the acid metabolites the phospholipid content of the cells in the stationary phase is equal to the cellular amount when the acid metabolites are not titrated. The phospholipid compositions during growth are also comparable in both cultures.

On the other hand, cultivation at a constant pH of 5.5 from the start has a significant effect on the phospholipid metabolism. The cellular amount of the total phospholipids in the stationary phase is only half of the value found in cells grown at non-constant pH. This decrease is due to a large decrease in the amount of lyso-15 per cell and also to a decrease of CL.

A rapid conversion of CL to PG has been found by shifting the pH from 6.8 to 8.0 during growth by addition of NaOH. Growth is inhibited immediately in the weak alkaline condition. The conversion of CL to PG may be due to the presence of an enzyme similar as the CL- specific phospholipase D which has been found in *H.parainfluenzae*.

The effect of pH on the phospholipid composition in the cultures devoid of human milk is similar as in the cultures with human milk. At a constant pH of 5.5 the cellular amount of lyso-15 is also decreased in comparison to the value found in a culture at a constant pH of 6.8. At pH 8.0 the initial relative amount of PG also constitutes at least 80% of the lipid phosphorus just as found in the presence of human milk at the same pH.

Cell wall synthesis is inhibited by lack of human milk and by some antibiotics. The cell wall inhibitors penicillin, cycloserine and vancomycin influence the cellular amounts of the phospholipids and the phospholipid composition as does lacking of human milk. In the presence of these antibiotics or in the absence of human milk the content of CL per cell is 4-6 fold increased and the total phospholipid content twice. In the presence of penicillin and vancomycin also a significant excretion of cellular phosphate has been observed.

In chapter 5 we have studied the turnover of the individual phospholipids. Pulse-chase experiments have suggested that PG can function as the phosphate donor in the synthesis of phosphogalactolipid compound-15. In view of the *sn*-glycerol 1-phosphate configuration in compound-15 a transfer of the unacylated glycerophosphate moiety of PG is likely.

PG is metabolically more active than CL and compound-15. During rapid growth a half-life time of PG is found of 90 min while growth continues for 0.5 duplication under this condition. However, this half life time has to be considered as a maximum value, since turnover of PG is disturbed more or less by a slow exchange of intracellular and extracellular phosphate. The slow exchange of inorganic phosphate across the membrane to the outside appears from ^{32}P-incorporation studies, for the increase of the specific activity of the phospholipids is proportional to the increase of cell mass.

It seems to us worthwhile to investigate whether the phosphogalactolipids in *B.bifidum* var.*pennsylvanicus* are involved in the synthesis of teichoic acids which contain galactose (see section 1.4). Another line of continued research might be to look for a relation between the phosphogalactolipids and a galactose transportsystem. If phosphoglycolipids will prove to be as common as glycolipids in nature, more investigation of the function of this class of lipids will be called for.

Het phosphogalactolipide component 15 dat onderzocht is in deze dissertatie is beschreven in de algemene inleiding als een phospholipide van taxonomische betekenis om *Lactobacilli* van *Bifidobacteria* te onderscheiden. De structuur van dit lipide is volledig opgehelderd voor de stam *Bifodobacterium bifidum* var.*pennsylvanicus* (Hoofdstuk 3). Het is echter niet uitgesloten dat het overeenkomende lipide in andere *Bifidobacteria* stammen slechts verschillen in de configuratie van het glycerophosphate- of het galactose deel van het molecule. Component 15 is een hoofdcomponent van de phospholipiden in *Bifidobacteria*, terwijl phosphoglycolipiden in andere bacterien in het algemeen in geringe mate voorkomen, uitgezonderd in *Mycoplasmas* (sectie 4.4).

De structuur van component 15 blijkt te zijn glycerophosphorylgalactosyldiglyceride met de glycerophosphaat helft in de *sn*-1 configuratie, de glycosidische binding in de β-configuratie en galactose voornamelijk in de furanose vorm. De basis structuur, glycerophosphorylgalactosylglycerol, is opgehelderd door quantitatieve analyse en door de analyse van de produkten verkregen door sterke alkalische hydrolyse van de gedeacyleerde component. Deze produkten bestaan uit glycerol, glycerophosphaat, galactosylglycerol en phosphorylgalactosylglycerol; de phosphaat bevattende produkten zijn overlappende brokstukken.

De furanose en pyranose isomeren van galactose bedragen 80% en 20% resp. van galactosylglycerol.

Het vóórkomen van phosphorylgalactosylglycerol zou suggereren, dat galactose in dit brokstuk uitsluitend aanwezig is in de furanose configuratie, maar dit blijkt niet juist te zijn. Het feit dat galactose ten dele in de pyranose configuratie voorkomt in dit brokstuk wijst op de waarschijnlijke vorming van een phosphodiester intermediair tussen koolstof atoom 3 of 4 en koolstof atoom 6 van galactose tijdens hydrolyse van de phosphodiester binding.

Tot dusver zijn alleen phosphoglucolipiden vermeld in de literatuur met glucose in de pyranose vorm. Uit deze componenten wordt bij alkalische hydrolyse geen produkt verkregen, waarin de phosphaat-glucose binding behouden is.

Glycerophosphorylgalactosylglycerol is ook gebleken de basis structuur te zijn van een lyso derivaat en van een geacyleerd derivaat van component 15, die beide voorkomen in *B.bifidum* var.*pennsylvanicus*, maar mogelijk verschillen de relative hoeveelheden van de furanose en de pyranose vormen van die in component 15.

In Hoofdstuk 4 hebben we bestudeerd het effect van sommige milieu factoren op de phosphogalactolipiden en op de andere membraan phospholipiden. Cardiolipine (CL), lyso-cardiolipine, component 15 en zijn lyso-derivaat worden gesynthetiseerd met een constante snelheid tijdens groei, in tegenstelling tot de synthese van phosphatidylglycerol (PG). De aanvankelijke netto toename van PG gaat geleidelijk over in een netto afname tijdens de groei. Vandaar dat de veranderingen in de relatieve hoeveelheden van CL, lyso-CL, component 15 en lyso-component 15 gedicteerd worden door de verandering in de hoeveelheid van PG.

De daling van pH van het medium tijdens de groei van pH 6.8 tot 5.2 als gevolg van suiker vergisting heeft geen significant effect op de phospholipide synthese. Wanneer de begin pH van 6.8 gehandhaafd blijft door titratie van de zure metabolieten is het phospholipide gehalte in de cellen in de stationaire phase gelijk aan de hoeveelheid per cel wanneer de zure metabo-

lieten niet getitreerd worden. De phospholipide samenstelling tijdens de groei is ook vergelijkbaar in beide culturen.

Aan de andere kant heeft het kweken van cellen bij een constante pH van 5.5 vanaf het begin wel een significant effect op het phospholipide metabolisme. De cellulaire hoeveelheid van de totale phospholipiden in de stationaire fase is slechts de helft van de waarde gevonden in cellen die gegroeid zijn bij niet-constante pH. Deze afname is te danken aan een grote afname van de hoeveelheid lyso-15 per cel en ook aan een afname van cardiolipine.

Een snelle omzetting van CL naar PG vindt plaats door een snelle verhoging van de pH van 6.8 naar 8.0 tijdens groei door het toevoegen van NaOH. Groei wordt onmiddellijk geremd in het zwak basische milieu. De omzetting van CL naar PG is mogelijk te danken aan de aanwezigheid van een enzym soortgelijk als het CL-specifieke phospholipase D dat gevonden is in *Haemophilus parainfluenzae*.

Het effect van de pH op de phospholipiden samenstelling in de cultures die verstoken zijn van moedermelk is soortgelijk als in de cultures met moedermelk. Bij een constante pH van 5.5 is de cellulaire hoeveelheid van lyso-15 eveneens afgenomen vergeleken met de waarde gevonden in een cultuur bij constante pH van 6.8. Bij een pH van 8.0 bedraagt de relatieve hoeveelheid van PG aanvankelijk ook minstens 80% van het lipide phosphor zoals ook gevonden is in de aanwezigheid van moedermelk bij dezelfde pH.

Celwand synthese wordt geremd door gebrek aan moedermelk evenals door sommige antibiotica. De celwand remmers penicilline, cycloserine en vancomycine beinvloeden de cellulaire hoeveelheden en de samenstelling van de phospholipiden op dezelfde wijze als het gemis aan moedermelk. In de aanwezigheid van deze antibiotica of in de aanwezigheid van moedermelk is het gehalte aan CL per cel 4-6 maal verhoogd en het totaal phospholipide gehalte 2 maal. In de aanwezigheid van penicilline en vancomycine is ook een aanzienlijke uitscheiding van cellulair phosphaat waargenomen.

In Hoofdstuk 5 hebben we bestudeerd de snelheid van synthese en afbraak "turnover" genaamd, van de afzonderlijke phospholipiden."Pulse-chase" experimenten zijn experimenten, waarbij radioactiviteit gedurende korte tijd door de cellen geincorporeerd wordt, waarna de ingebouwde radioactiviteit in de cellulaire componenten weer wordt verdreven. Dit soort experimenten hebben gesuggereerd, dat PG als phosphaat donor kan fungeren voor de synthese van het phosphogalactolipide component 15. Gezien de *sn*-glycerol 1-phosphaat configuratie in component 15 is een overdracht van de ongeacyleerde glycerophosphaat helft van PG waarschijnlijk.

PG is metabolisch actiever dan CL en component 15. Tijdens snelle groei is een halfwaarde tijd voor PG van 90 min gevonden, terwijl de groei van de cellen overeenkomt met een 0.5 verdubbeling onder deze conditie. Deze halfwaarde tijd moet echter beschouwd worden als een maximale waarde omdat "turnover" van PG vertroebeld wordt in meer of mindere mate door een langzame uitwisseling van intracellulair- en extracellulair phosphaat. Dat anorganisch phosphaat langzaam wordt uitgewisseld door de membraan naar buiten blijkt uit ^{32}P-incorporatie studies waarbij de toename van de specifieke activiteit van de phospholipiden evenredig is met de toename van de celmassa.

Het lijkt ons de moeite van het onderzoeken waard om na te gaan of de phosphogalactolipiden in *B.bifidum* var.*pennsylvanicus* betrokken zijn bij de synthese van teichoine zuren die galactose bevatten (sectie 1.4). Een andere lijn van voortgezet onderzoek zou kunnen zijn het onderzoek naar een relatie tussen de phosphogalactolipiden en een galactose transportsysteem. Indien zal blijken, dat phosphoglycolipiden even algemeen voorkomen in de natuur als glycolipiden, zal er meer melding gemaakt worden van onderzoek naar de functie van deze klasse van lipiden.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 13 april 1944 geboren te Driebergen. Het einddiploma Gymnasium-b werd behaald in 1963 aan het Revius-Lyceum te Doorn. In datzelfde jaar begon hij met de scheikunde studie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen, letters f en g, werd afgelegd in 1967; het doktoraalexamen met als specialisatie biochemie en als bijvak microbiologie in 1970.

In april 1970 begon hij zijn werkzaamheden aan het Laboratorium voor Biochemie van de Medische Faculteit van de Katholieke Universiteit te Nijmegen. Vanaf 1 oktober 1974 zal hij verbonden zijn d.v. aan het Laboratorium voor Gynaecologie van dezelfde Faculteit.

STELLINGEN

1. De betekenis van het onderzoek omtrent de regulerende functie van cyclisch AMP bij bacteriegroei ten behoeve van kankeronderzoek is tot nu toe te weinig onderkend. (P.Emmelot, Europ.J.Cancer,*9*,(1973), 319; E.M.De Robertis et al., Biochem.Biophys.Res.Commun.*55*,(1973), 758).

2. Remming van DNA synthese o.i.v. actinomycine D gaat gepaard met een sterke toename van tyrosine transaminase activiteit in rattelever. Bij het verklaren van dit fenomeen houden T.D.Gelehrter en G.M.Tomkins (J.Mol.Biol.,*29*,(1967),59) geen rekening met de mogelijkheid dat de remming van enzymsynthese kleiner is dan de remming van enzym afbraak.

3. Recent ontwikkelde geavanceerde hybridisatie-technieken maken het mogelijk te zoeken naar mRNA, dat het transcript is van repeterend DNA. Met uitzondering van histon-mRNA is zulk mRNA nog niet gevonden. Het verdient aanbeveling dergelijk onderzoek thans te richten op een organisme - zoals gist -, waarvan op genetische gronden vermoed wordt, dat het zulk mRNA bevat. (Klein et al., Proc.Nat.Acad. Sci.U.S.,*71*,(1974), 1785; Ten Berge et al., Molec.gen.Genet.,*123*. (1973),233).

4. Bij de bepaling van pyruvaat-carboxylase activiteit dient men te voorkomen dat de concentratie van de effector acetyl-CoA de beperkende factor wordt bij de reactie. (M.F.Utter en D.B.Keech, J.Biol. Chem.,*238*,(1963),2603).

5. Ten onrechte wordt gesuggereerd, dat de nauwkeurigheid van een radio-ligand test vergelijkbaar is met die van een radio-immunotest. (S.Kammerman et al., Endocrinology,*91*,(1972),65).

6. Microradiografische bepalingen van eiwitgehalten in ooglens-coupes geven te hoge uitkomsten. (B.Philipson, Invest.Ophthalmol.,*8*,(1969), 258).

7. De Katholieke Universiteit dient haar taak als bijzondere universiteit serieus te nemen door voortdurend een appèl te doen op haar medewerkers om hun wetenschapsbeoefening te integreren in een christelijke levensvisie. (E.C.F.A.Schillebeeckx et al., Annalen van het Thijmgenootschap,59,(1971),no.1).

8. Jom Kippoer, de Joodse feestdag van de verzoening, zal een wijdere betekenis krijgen die ook de relatie tussen Christenen en Joden zal omvatten, wanneer de existentiële verbondenheid van de Joden met Jeruzalem erkend wordt door Christenen met woord en daad.

9. Het nuttige effect voor de maatschappij van veel laboratoriumonderzoekingen wordt onnodig verkleind of wordt zelfs negatief wanneer het chemisch afval nonchalant wordt weggespoeld door de gootsteen. (Milieubedreiging .... Leven of dood, M.Basilea Schlink).

10. Het vaak zeer belangrijke aandeel van analisten bij het tot stand komen van een proefschrift kan te weinig tot uiting gebracht worden door een korte vermelding in de lijst van dankbetuigingen.

Stellingen behorende bij het proefschrift van F.W. van Schaik